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MICRODETERMINATION OF A Δ^{4}-3 KETOSTEROID (PROGESTERONE) OBTAINED FROM SMALL VOLUMES OF SERUM[1]

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AT THE PRESENT time, the qualitative and quantitative estimation of progesterone in tissues and body fluids depends upon biological tests. These have proved most useful since, by their specificity and quantitative character, the identification, isolation and synthesis of progesterone was possible (1–6). Moreover, much of the physiology of sex and reproductive cycles is understood today because of the uses to which the biological tests for progesterone have been put (6).

The basic method of Corner and Allen (1, 3) has been modified in minor respects by Clauberg (7) and by McPhail (8), but only with respect to the manner of conducting the test, and without improving appreciably its sensitivity. Topical application of progesterone to the endometrium (9) provides a highly sensitive test for the qualitative determination of progesterone, but the endometrial responses, or absorption of the hormone, or both, are too variable in this test to permits its use for quantitative purposes (10, 11).

The value of the biological methods of assay, however, suffers an inherent limitation, namely, that a relatively large amount of progesterone is required for a test. Thus, for a single response of maximal progestational proliferation of the rabbit endometrium by the Corner-Allen procedure, about one mg. of hormone is necessary, and for material of unknown progesterone-content, two or more rabbits are required. Accordingly, a more sensitive method of quantification is needed for the solution of many physiological, embryological, and clinical problems.

Other methods for assaying the sex and related steroid hormones have been devised. In general, it may be said that these are colorimetric tests whereby 17-ketosteroids are measured (12–22). These depend upon the Zimmerman reaction (23), combining the hormone with a m-dinitrobenzene in the presence of alkali, yielding a color reaction which is read photometrically. Results of this method compare favorably with biological tests (12, 20); in urine extracts, they have been used to detect minute amounts of 'sterone' (12). But these methods

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do not, of course, lend themselves readily to the measurement of progesterone; on the contrary, they provide a means for distinguishing 17-ketosteroids from 20-ketosteroids under suitable experimental conditions (12, 20, 24, 25, 26). Tastaldi and Campos (27) and McCullagh, Schneider and Emery (26) have utilized the Zimmerman reaction for detection of pure desoxycorticosterone acetate, but not in extracts containing the substance. Holtorff and Koch (20), however, obtained practically the same extinction for testosterone and desoxycorticosterone. Esterification of 20-ketosteroids, it appears, renders these substances susceptible to this type of analysis (27).

A more promising method for the detection of small quantities of progesterone is the polarographic method of Heyrovský (28) as adapted by Wolfe, Hershberg and Fieser (29) and Wolfe, Fieser and Friedgood (30). The latter authors have established that Δ^4-3 ketosteroids are distinguishable from others, even from Δ^1-3 ketosteroids. As yet, however, the method has been developed only for the analysis of urinary substances, with a sensitivity of about 0.05 mg. per cc. of extract. For problems of physiological importance where progesterone is concerned, this is not sufficiently sensitive. Müller, who has used the polarographic method in other types of work (31, 32, 33), believes that the method could be developed to the point where 0.001 mg. of hormone may be detected with an error of 25 to 50 per cent, and the estimation of 0.005 mg. of hormone, with an error of 10 per cent (personal communication). This, however, is a prediction for the method which has yet to be verified.

In casting about for other sensitive and highly specific technics, that of absorption spectroscopy looked promising. The infra-red portion of the spectrum would be most specific, since it depends upon molecular rotation-vibration spectra, at least in wave-lengths of 1 to 20 μ (34). Work with steroids bears out this statement (35), but infra-red spectroscopy is suited as yet only to qualitative work. In the ultraviolet region of the spectrum, selective absorption is effected by chromophores which are resonating groups involving two or more atoms coupled by sharing four, rather than two electrons (34). A particularly strong and characteristic group is that of α, β unsaturated ketones (R $-\overset{\overset{O}{\|}}{C}-\overset{|}{C}=C<$) which has an absorption maximum at 240 mμ. It is this characteristic which originally led Allen and Wintersteiner (5) to assign to ring I of the progesterone molecule the α, β unsaturated ketone configuration that is now generally accepted. Ample work has been carried out to prove that this is an identifiable tag which distinguishes Δ^4-3 ketosteroids from many others under suitably controlled conditions of extraction and measurement. The basis for this statement rests upon the establishment of absorption spectra for a variety of steroid substances (36–45).

Woodward (44) has accorded this view a critical evaluation which led the present writers to the belief that, if one employed a suitable method of purification, one could obtain significant data. Thus, if *a*), neutral, non-volatile compounds of high molecular weight are separated, *b*), the chromogenic activity of the extract is concentrated in the ketonic fraction, and *c*), the absorption maximum is at 240 mμ and typical of α,β unsaturated ketones, one can be reasonably certain that he is dealing with Δ^4-3 ketosterones in the same manner, and to the same degree that Callow, Callow and Emmens were in their use of the Zimmerman reaction for the detection of 17-ketosteroids. And by combining ultraviolet absorption technic with the colorimetric reaction for 17-ketosteroids, one could further differentiate between progesterone, Δ^4-cholestenone, and cortical hormone and a limited number of related substances on the one hand, and testosterone, androstenedione and similar related Δ^4-3,17 ketosteroids on the other. Only with the polarographic method have greater claims been made for distinction between closely related steroid molecules (29). With this assurance, a sensitive method is open to us, inasmuch as ultraviolet absorption spectrophotometry may be carried out with very dilute solutions, of the order of 0.3×10^{-4} molar concentration. The limitations and greater variations would appear to be largely on the side of the chemical extraction procedures. This paper presents in a preliminary

manner the pattern of such a method, and the nature of the results which may be expected of it.

EXPERIMENTAL PROCEDURES AND RESULTS

The first part of this work is concerned with *a*), the aspects of the absorption spectra of selected, pure steroid substances, and *b*), the relation between the concentration of progesterone and estrogens in alcoholic solutions and the absorption of monochromatic light. The second part is concerned with the addition of these substances to small amounts of serum in known concentrations, with subsequent extraction and determination spectrophotometrically of the quantity of hormone or hormones recoverable. Identical photometric conditions were maintained throughout both parts of the work.

All measurements were made in a large Hilger quartz spectrograph. The region of the spectrum recorded on ultraviolet sensitized plates (Eastman 103-0, 4×10 inches) was from 224 to 280 mμ. Five exposures were made on each plate, one of which was of the iron spectrum alone, another, the iron spectrum through alcohol. The light source was an iron spark (25,000 volts). To permit easy measurement of the extinction coefficient without photoelectric densitometry, a seven-step logarithmic rotating sector was employed. The plates were analyzed by matching extinction values according to the recommendations of Brode (34). Crystalline hormones,[3] which were not recrystallized, were used.

The hormones were weighed on a Roller-Smith precision balance in 5-mg. quantities. These were then made up to 2 mg. per cc. in aldehyde-free absolute ethyl alcohol. All dilutions were made with the aid of a tuberculin syringe, a clean one being used for each dilution. All determinations were made in Bausch and Lomb quartz absorption cylinders of 1 cm. length, and with a capacity of 2.5 cc. each.

Part I. (a) Absorption of Pure Steroids

The problem of the absorption of ultraviolet light by pure steroidal substances did not require extensive study, since the literature contains ample evidence of this property of each of the substances used (see above). It was sufficient to ascertain that, in our hands, each of the substances would yield data which were consonant with those found by other investigators, notably, Hogness, Sidwell and Zschiele (36), Woodward (44), Menschick, Page and Bossert (46) and others. This expectation was realized and the results are summarized in table 1. These data bear out the view of Woodward, in a critical evaluation of the literature, that an absorption maximum at 240 mμ is typical of Δ^4-3 ketosteroids.

The estrogens, of all the substances examined, are in a class by themselves, since they exhibit two absorption maxima, one in the region of 220 to 230 mμ, the other in the region of 280 mμ. This is in agreement with published data with respect to these compounds (36, 42, 43, 45). The curves of the molecular extinction values for estrone, estriol and progesterone are shown in figure 1, along with those of two Δ^5-pregnene 3-ol, 20-one substances. From this, it is clear that the point of maximum light absorption by progesterone straddles part of the region of low absorption between the two absorption maxima for both estrogens. Estradiol, equilin and equilinin all have ab-

[3] The hormones were supplied by the following firms: Ciba Pharmaceutical Products, Summit, N. J.: progesterone; Δ^5-androstenediol 3, 17; Δ^5-androstene 3-ol, 17-one; 21-acetoxy Δ^5-pregnene 3-ol, 20-one; Δ^5-pregnene 3-ol, 20-one: Δ^4-androstenedione, 3, 17; methyl testosterone; testosterone; testosterone propionate; desoxycorticosterone acetate; estradiol; cholesterol.

Parke, Davis and Co., Detroit, Mich.: estrone, estriol.

Schering Corp., Bloomfield, N. J.: Δ^4-cholestenone; progesterone; testosterone propionate; cholesterol acetate; estradiol benzoate.

TABLE 1

Steroid	Concentration	Absorption Maximum	Molecular Extinction
	mg./cc.	mμ	log E
Progesterone	0.02	242	5.85
Δ^4-Androstenedione, 3, 17	0.02	245	5.38
Δ^4-Cholestenone, 3	0.02	242	5.42
Desoxycorticosterone acetate	0.02	240	5.57
Testosterone	0.02	242	5.30
Methyl testosterone	0.02	240	5.32
Testosterone propionate	0.02	242	5.30
Δ^4-Androstene-3(B)ol, 17-one	0.05	0	0.00
21 Acetoxy, Δ^5pregnene 3(B), 20-one	0.05	0	0.00
Δ^4-Androstenediol 3(B), 17(trans)	0.05	0	0.00
Δ^5Cholestene-3 ol	0.05	0	0.00
Δ^5pregnene-3(B) ol, 20-one	0.05	0	0.00
Δ^5pregnene-3(B) ol, 20-one acetate	0.05	0	0.00
Cholesterol	0.05	0	0.00
Cholesterol acetate	0.05	0	0.00
Estrone	0.02	280	4.90
Estradiol	0.02	280	4.80
Estradiol benzoate	0.02	280	4.70
Estriol	0.02	280	4.72

sorption curves indistinguishable from estrone and estriol. One may not use ultraviolet absorption spectrophotometry, therefore, to distinguish between them. The fact that one may easily differentiate between the estrogens and progesterone is a most convenient circumstance, however, since it offers a ready means of simultaneously detecting and measuring the quantity of each type of substance in a solution. The import of this fact is further heightened by the circumstance that the most efficient methods used for the extraction and purification of progesterone from tissues do not permit its separation from estrogens without considerable loss of the former (4).

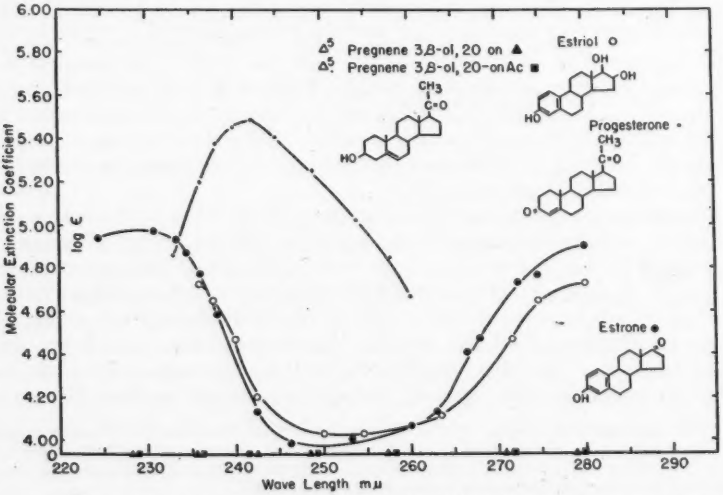

Fig. 1. MOLECULAR EXTINCTION VALUES for 5 ketosteroids, estriol, estrone, progesterone, Δ^5 pregnene-3β-ol, 20-one. Note that the maximum absorption for progesterone (typical of α, β ketones) straddles the region of minimal absorption between the maxima for estrogens.

(b) *Concentration-Extinction Relationship with Progesterone and Estrogens*

The effect of concentration of the hormones upon ultraviolet absorption is shown in figures 2 and 3. Here, it may be seen, a high degree of sensitivity (i.e., maximum extinction with a minimum change in concentration of substance) is observed between 0.005 and 0.010 mg. of progesterone per cc. A more sensitive method (photoelectric densitometry) would doubtless show that lower concentrations of progesterone are detectable with equal sensitivity. We have not had an opportunity to examine this point in detail. It is, consequently, a matter which urgently requires study.

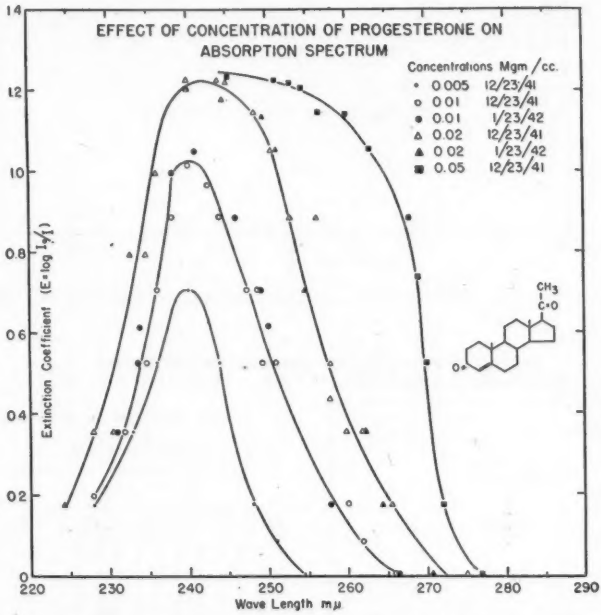

Fig. 2. Effect of concentration of progesterone on extinction of monochromatic light.

The effect of estrogen concentration on the extinction of light is shown in figure 3. The region of greatest sensitivity for this substance lies between 1 to 50 μg. per cc. As noted above (see fig. 1), no distinction can be made in the spectral range employed between the several estrogens, estrone, estradiol and estriol. This confirms the work of Mayneord and Roe (45) and of Hogness, Sidwell and Zschiele (36). The latter authors call attention to the fact that the chromophore for the estrogens (Ring I), which bears a structural resemblance to p-cresol, has an almost identical absorption spectrum in the ultraviolet with that of this substance. Because of the presence of cresols in urine concentrates, these investigators state that ultraviolet absorption spectrophotometry may not be used for the determination of estrogens in such extracts. Dobriner, Lavin and Rhoads (37) rightly point out, however, that these extracts were crude, and could have been further purified. This same criticism, Dobriner, Lavin and Rhoads apply to other workers who have failed in the application of ultraviolet absorption spectroscopy to quantitative work in biological problems.

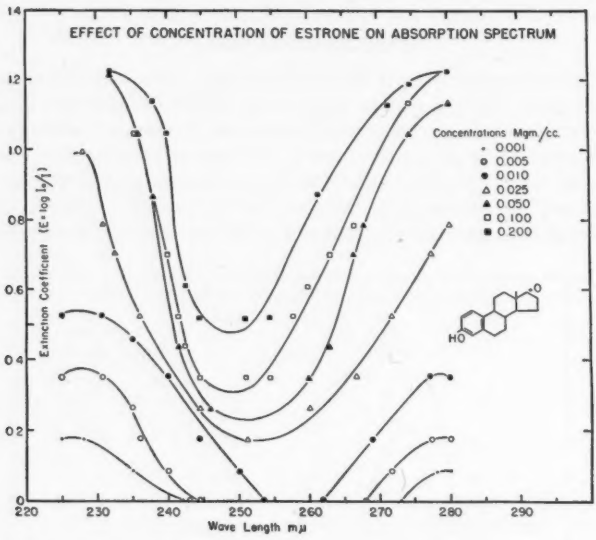

Fig. 3. Effect of concentration of estrone on extinction of monochromatic light.

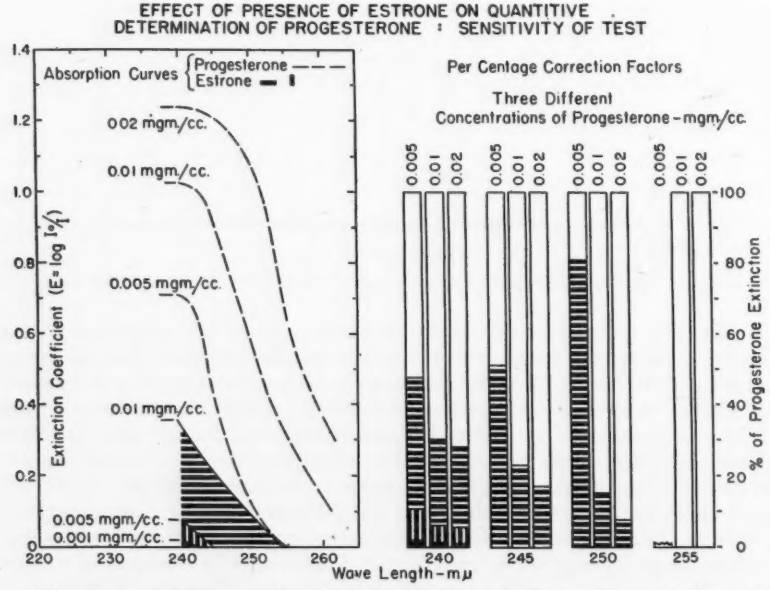

Fig. 4. Influence of estrogen at 3 levels of concentration (0.001–0.01 mg. per cc.) when determined simultaneously. At the right, portions of the respective curves between 240 and 260 mμ are shown as proportions of the progesterone data. From this, the value of reading progesterone absorption at 245–250 mμ instead of 240 mμ, is clear, since only at the very high estrogen values of the order of 0.01 mg. of estrone per cc. contribute to the extinction of light in this region.

For the detection of progesterone, however, the significance of the fact that this hormone exhibits an absorption maximum at 240 mμ, and that this lies between the absorption maxima for estrogens (and the cresols), is clear; the luteal hormone may be measured simultaneously in the presence of these other substances. This has been done by combining the curves of progesterone and estrogen absorption spectra between 240 and 255 mμ, as shown in figure 4. At the left in this figure the estrogen curves are indicated by barred areas, the progesterone curves by dotted lines. At the right, the proportion (percentage) which estrogen-extinction bears to progesterone-extinction is shown for 4 different wave-lengths at 3 different levels of progesterone concentration. Consideration of the relationship shown here, and outlined above, will suggest to the reader that, by ascertaining the concentration of estrogen in a given solution between 265 and 280 mμ (where progesterone absorption does not occur;

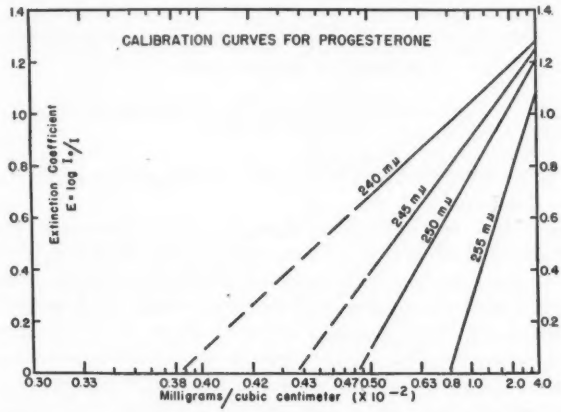

Fig. 5. Calibration curves for progesterone, at 240–255 mμ. The broken lines are extrapolations of the solid lines, beyond the regions determined by experimentation.

see figure 1) the quantity of progesterone may be determined by reading the difference between concentration of this hormone and the total extinction values read at 240, 245, 250 and 255 mμ, particularly the last three. The difference so obtained, if the curve be characteristic for progesterone (at a suitable dilution which must be determined by trial and error), may then be read off the calibration curve for progesterone (fig. 5) and the concentration of progesterone in that particular dilution so determined. In addition to the calibration curve for progesterone (fig. 5), the calibration curve for estrogens at 265, 270 and 275 mμ is shown in figure 6, A. For convenience, the extinction values of varying concentrations of estrogen at 240, 245, 250 and 255 mμ are shown in figure 6, B (inset). The procedure just described is that employed in the recovery of these hormones from serum to which they had been added separately, as well as combined, in the account which follows.

Part II. Recovery of Progesterone and Estrogen from Serum

For this work, blood was drawn from unanesthetized rabbits by heart puncture. Four rabbits were used, 3 of which were castrated, the fourth was in the 30th day of pregnancy. The blood was allowed to clot, the clot broken and centrifuged, and the serum divided into aliquots of 10 cc. In no case was the serum free of some hemo-

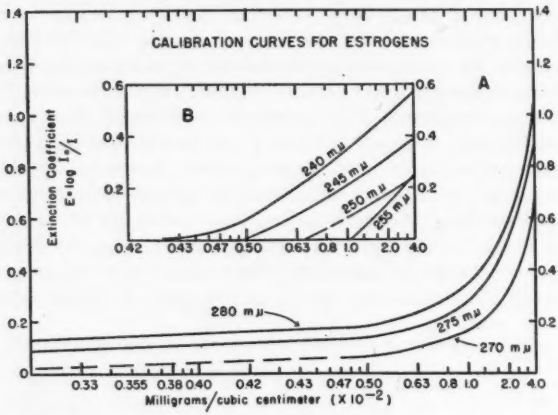

Fig. 6. CALIBRATION CURVES FOR ESTROGENS, *A*, between 270–280 mμ; *B*, between 240–255 mμ.

globin from laked erythrocytes. The number and disposition of the several blood samples were as noted in table 2.

From this table, it will be observed that 26 serum samples were extracted. Four were blank controls of serum only, 11 were with progesterone only, 4 with estrogen only, and 6 samples contained both hormones. Of these extractions, some were unsatisfactory for spectrophotometric analysis owing to too great general absorption throughout the region of the spectrum examined. These were limited to *rabbit* C2 in which the hormone was added to the serum in corn oil and were fractions *B*, C and *E*. Accordingly, 23 successful extractions were carried out, 8 with progesterone alone. The procedure for extraction of the hormone was as follows.

Recovery of hormones from serum. Addition of the desired kind and quantity of hormone in 0.5 cc. of ethyl alcohol (absolute) or 1 cc. of corn oil (*rabbit* C2) was made

TABLE 2. HORMONES[1] ADDED, MILLIGRAMS PER CUBIC CENTIMETER

Rabbit	Serum Only	*B*	*C*	*D*	*E*	*F*	*G*	*H*
C1	A	Estradiol 0.20	Progest. 1.0	Progest. 0.10				
C2	A	Progest. 0.20	Progest. 0.10	Progest. 0.02	Progest. 0.01	Progest. 0.002		
C3	A	Progest. 0.02	Progest. 0.01	Estriol 0.10	Estriol 0.025	Progest. 0.02 Estriol 0.10	Progest. 0.02 Estriol 0.05	Progest. 0.02 Estriol 0.025
C4	A	Progest. 0.02	Progest. 0.01	Estriol 0.10	Estriol 0.025	Progest. 0.02 Estriol 0.10	Progest. 0.02 Estriol 0.05	Progest. 0.02 Estriol 0.025

Note: Hormones added in 0.5 cc. of alcohol, except in case of *rabbit* C2, in which hormone was contained in 1 cc. of corn oil.

[1] Progest. = progesterone.

to the respective serum samples. These were agitated for 30 to 60 seconds, and then double the volume (20 cc.) of 95 per cent ethyl alcohol was added and the whole shaken again until thoroughly mixed. The white protein precipitate portion was removed by centrifugation, and procedures (see below), based on W. M. Allen's successful method for the extraction of purified progesterone from large quantities (1.5 kg.) of sow corpora lutea (4) were followed. The volumes of solutions were arbitrarily reduced, roughly in proportion to the smaller amount to be extracted. Another modification was the use of high vacuum in each of the distillation steps in order to minimize the possibility that α,β unsaturated ketones of low molecular weight (such as mesityl oxide, or equilibrium compounds with aceto-acetic acid) would remain behind to affect the spectrophotometric determination.

Outline for extraction of hormones from serum. Serum, obtained from unanesthetized rabbits by heart puncture, was divided into 10 cc. aliquots. The kind and amount of hormone to be extracted for spectrophotometric analysis was added. Two volumes of 95 per cent ethyl alcohol were added, and the mixture centrifuged.

Precipitate (a)	Supernatant (b)
Proteins (discarded)	Reduced in high vacuum at 50–60°C. to *ca.* 1 cc. Washed and decanted 4 times with 15 cc. portions of anhydrous ethyl ether (peroxide free).
Ether-soluble (c)	Ether-insoluble (d)
All fatty substances. Reduced *in vacuo* at 40°C. to 1–2 cc. Added 8–10 cc. acetone with 1–2 drops saturated alcoholic $MgCl_2$. Filtered through Munktells paper; washed with acetone	Protein, split-products (discarded)
Acetone-soluble (d)	Acetone-insoluble (e)
Distil in vacuo at 50–60°C. Extracted with boiling methyl alcohol (70%). Chilled at −4°C. for 12 hours. Filtered while cold.	Phospholipids (discarded)
Cold methyl alcohol-soluble (f)	Cold methyl alcohol-insoluble (g)
Distil to 0.5 cc. Take up in 5 cc. absolute ethyl alcohol (aldehyde-free). Ampuled until diluted and examined spectrophotometrically.	Neutral fat and cholesterol; (discarded)

General absorption in extracts. The general absorption of serum-controls in the spectral range examined is shown in figure 7. All the extracts, except C_4, showed such marked absorption at one-half the original concentration of serum, that no hormone absorption curves were determinable at this dilution. By further dilution to one-tenth the original serum-concentration, determinations were possible with each extract, except in the case of C_2 as noted above. With C_4, readings were easily possible at a serum dilution equivalent to one-fourth. This means that, since progesterone was detected in a number of cases at 0.002 to 0.003 mg. per cc. of alcohol the maximum sensitivity for this hormone was of the order of 0.008 to 0.010 mg. per cc. of original serum. For estrogens it was of the order of 0.004 mg. per cc. of alcohol.

Careful review of the details of the separate extractions and spectroscopic determinations, suggests that improvement in the sensitivity of the test lies in the direction of reducing or eliminating general absorption by better chemical technic so that lower serum dilutions, or better, serum concentrates may be accurately assayed spectrophotometrically. General absorption, it should be noted, does not alter the accuracy

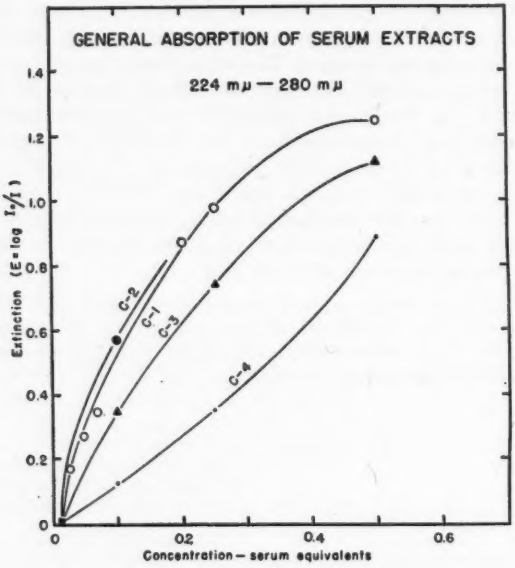

Fig. 7. General absorption of serum extracts. For greater sensitivity, rendering determinations possible at higher serum concentration equivalents, this must be reduced by more efficient chemical procedures. *Extract* C_4 showed considerable improvement over the others.

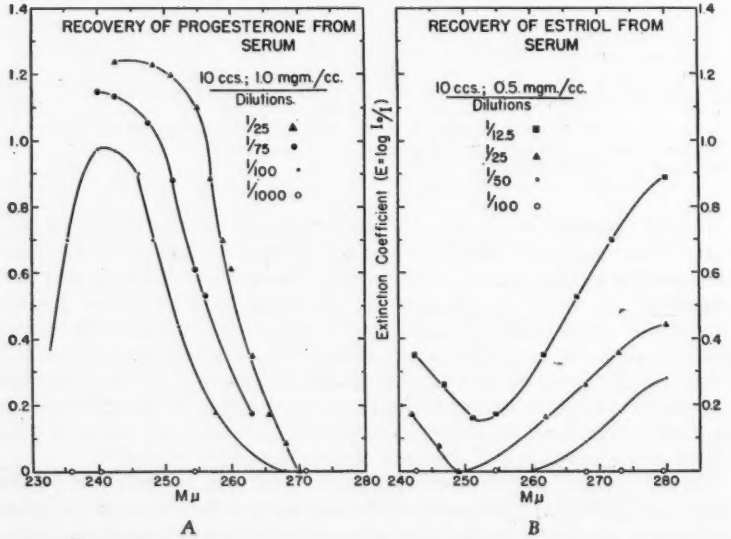

Fig. 8. A (*left*), Recovery of progesterone from 10 cc. of serum. One mg. per cc. added originally. *B* (*right*), recovery of estriol from serum, 0.5 mg. per cc. originally added. See table 3 for data on the recovery of smaller amounts of these hormones.

TABLE 3. HORMONE EXTRACTIONS FROM 10 CUBIC CENTIMETERS OF SERUM

Number of Dilutions Examined	Amount of Progesterone Present mg/cc.	Amount of Progesterone Recovered, mg/cc.	Per Cent Recovered	Per Cent Error	Corner-Allen Test	
					Serum equivalents cc.	Endometrial proliferation
a. Progesterone extraction						
23	0.000	0.000	0			
3	0.002	0.000	0	−100		
4	0.010	0.012	120	+ 20		
4	0.020	0.022	112	+ 12		
2	0.020	0.027	135	+ 35		
2	0.100	0.117	117	+ 17		
4	0.100	0.109	109	+ 9		
4	0.200	0.180	80	− 20		
4	0.500	0.395	79	− 21	2.4 1.6	++ 0
6	1.000	0.986	99	− 2	1.4 0.8	+++ 0
b. Estrogen extraction						
23	0.000	0.000	0			
3	0.025	0.028	116	+16		
4	0.100	0.071	71	−29		
3	0.200	0.192	96	− 4		
5	0.500	0.395	79	−21	2.4	0

c. Progesterone and estrogen recovered from serum

Number of Dilutions Examined	Hormones Present		Estrogen Recovered		Progesterone Recovered	
	Progesterone	Estriol	Amount	% Error	Amount	% Error
23	0.000	0.000	0.000		0.000	
3	0.020	0.025	0.026	+ 6	0.021	+ 7
3	0.020	0.050	0.555	+11	0.024	+23
4	0.020	0.100	0.553	−45	0.028	+40
6	0.500	0.500	0.456	−11	0.416	−17

with which the absorption curves may be read. Thus, the percentage recovery of hormones, described below, bore no relation to the dilution of the extract for any of the measurements made, when comparisons between a hormone-containing extract and a serum-blank were in any way possible. Nevertheless the fact remains that in the work as thus far carried out, the principal bar to higher sensitivity lay in the direction of insufficient elimination of substances in the extracts which give rise to too general absorption, unless reduced in amount by dilution.

Hormone content of serum-blanks. The results of the above procedures, applied to the extraction of progesterone and estrogens as outlined in table 2, are given in table 3, *a*, *b* and *c*.

In none of the serum-controls examined was there evidence of either progesterone, or other α,β unsaturated ketosteroid, or of estrogen. This means that, in view of the limit of sensitivity of the method as used in this work, such substances, if present, were in less concentration than 0.008 to 0.010 mg. of progesterone-equivalent per cc. of original serum and 0.004 mg. estrogen per cc. Since 3 of the rabbits were castrated many months previously, this result is not surprising and no significance should be

attached to an extraction of the serum from a single rabbit taken on the 30th day of pregnancy. Moreover, Albrieux (47) has adduced evidence to show that estrogens are carried preponderantly in the erythrocyte fraction of blood and least in the serum.

Recovery of progesterone. The recoveries of progesterone are summarized in table 3, *a*. An example of the absorption curves obtained in one case is shown in figure 8, A. Throughout the range of concentrations studied (0.002–1.0 mg. of progesterone per cc.), the lowest concentration was, for the reasons outlined in the paragraphs on

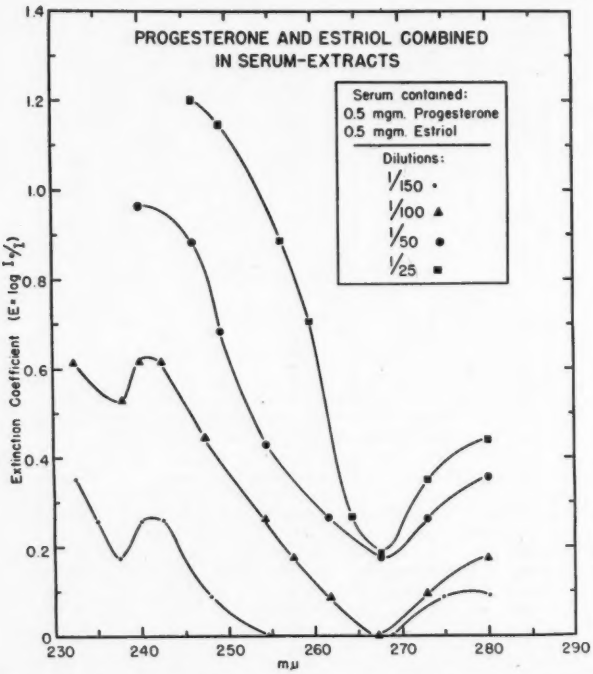

Fig. 9. Recovery of progesterone and estriol simultaneously from a serum sample of 10 cc. See text for description, and table 3 for data on other similar extractions.

general absorption of extracts, not detectable. The rest were recovered with the errors shown in the table.

In two cases only was there enough extract with sufficient progesterone remaining from the spectroscopic work for a bioassay, as estimated from the quantity of progesterone originally added to the serum. These were carried out as follows: in one test, a rabbit received the equivalent of 0.8 mg. of progesterone in the original serum in the standard Corner-Allen procedure, the second rabbit, an equivalent of 1.2 mg. of progesterone. In the second test, the rabbits received, respectively, the equivalent of 0.8 and 1.4 mg. The evaluations of the tests, kindly made by Dr. Corner, were:

Test 1	0.8 mg.	equivalent	0	progestational	proliferation
	1.2 mg.	equivalent	++	progestational	proliferation
Test 2	0.8 mg.	equivalent	0	progestational	proliferation
	1.4 mg.	equivalent	+++	progestational	proliferation

This means that, as nearly as one may say, the first test showed ≤ 48 per cent recovery, the second test, ≤ 72 per cent recovery of the hormone. These compare with 79 per cent and 98.6 per cent, respectively, for the spectroscopic tests. If more of the extracts had been available for bioassay, far more adequate tests would have been possible.

Recovery of estrogens. The data on the recovery of estrogens are summarized in table 3, *b*. Recoveries were obtained with about the same error as in the case of progesterone. Partial absorption curves for one determination are shown in figure 8, B. A Corner-Allen test, made as a control for the one noted in table 3, *a*, yielded a negative test for progesterone; no biological test for estrogen was carried out.

Recovery of progesterone and estrogen simultaneously. These data are summarized

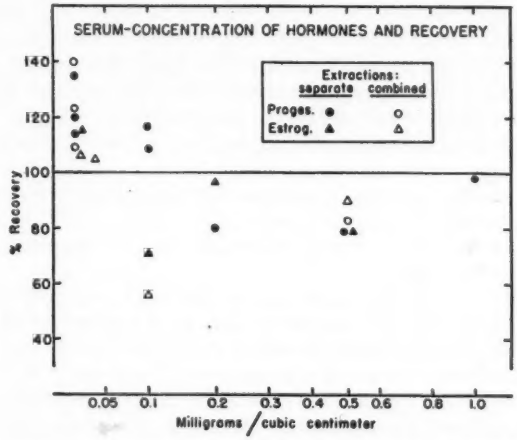

Fig. 10. INFLUENCE OF THE CONCENTRATION of hormones on percentage recovery of the hormones. With 0.05 mg. per cc., the values tend to be too high; with more than 0.1 mg. per cc., too low. See text for discussion.

in table 3, *c*, and an example of the absorption curves is shown in figure 9. It is clear from the data in table 3 that each of the hormones was recovered with about the same accuracy as when they were extracted and measured separately.

An essential part of this determination is shown in figure 9. When the hormones are present in too great concentration, the absorption curve is not characteristic for Δ^{4}-3 ketosteroids; it shows two maxima, and the contour of the curve from 240 to 265 mμ does not resemble that of progesterone, alone. This is so since, for the high extinction coefficient obtained at 240 mμ, the curve is a composite of absorption from both progesterone and estrogen. Only by diluting the extracts to a point where the maximum at 240 mμ becomes clearly evident may one feel assured that the α,β unsaturated ketone is present. This was found in three of the extracts studied, but not in the fourth. Since only known hormone concentrations were dealt with, however, the computations were made in this case anyway. This would not have been possible with extracts of unknown hormonal origin.

DISCUSSION

The comments to be made regarding the foregoing results are several. These bear upon the percentage error of the results, the inadequacies of the work as at present

carried out, and the general limitations of the technic which circumscribe the uses to which it may be successfully applied.

The errors that were found in this small series of data are not large. For the most part, they were of the order of 10 to 25 per cent, with three exceptions. Two of these (see table 3, c) occurred in one fraction, suggesting that some condition of the extraction or measurement may be at fault. The data do not show whether the error is in the extractions or the determination. That it may well be in the latter is indicated by the fact that when the concentration of either one of the hormones in the original serum is 0.02 to 0.05 mg. the apparent recovery exceeds 100 per cent, and with greater concentrations of hormone, recovery is less than 100 per cent. This is shown in figure 10. The error appears to be systematic, but the reason for this is not known.

The limitations of the spectrophotometric method as used in this study have been cited above, as they occur in the several phases of the work. The chemical aspect is one which should yield readily to greater experience and more efficient procedures. The solution of this will probably reveal the true limits of sensitivity of the spectrophotometric technic, for reasons which have already been described. When, however, the methods of extraction and analysis are perfected to their utmost, there will yet be inherent limitations in the uses to which ultraviolet absorption-spectrophotometry may be put. This is necessarily so since all Δ^4-3 ketosteroids are measured. By appropriately combining the data obtained by this method, however, with those for estimation of 17-ketosteroid determinations on the same sample as stated at the outset, one might correct for some of the Δ^4-3,17 ketosteroids. But this is a matter that remains to be tested experimentally.

If, then, there are inherent limitations in ultraviolet absorption spectrophotometry owing to lack of complete specificity, what are the uses to which it may be applied with sound scientific logic? Concisely stated, the answer must be: to those experimental conditions in which it is certain, or highly probable, that one is dealing with progesterone. This applies, with equal force, to use of the method in investigations involving testosterone or one of its esters, to androstendione (Δ^4-3,17-one), and to adrenal cortical hormones. Examples of such problems that come to mind are numerous. These include, the fate of any of these substances in blood, *in vitro* as well as *in vivo;* the metabolic conversion (utilization) of specific hormones by the liver, uterus or other organs; the rate of production of these hormones by glands, such as the testis, adrenal gland or corpus luteum by direct recovery from the venous blood of these organs in animal experiments. This list could be much extended, but always with the reservation in mind that to merely make a simple determination for Δ^4-3 kestosteroids on a fluid or tissue, is no test at all for progesterone, or for testosterone, or for an adrenal cortical hormone. The method is not, therefore, useful for evaluation of clinical material without further development.

SUMMARY

1. A survey is made of the biological, colorimetric, and potentiometric methods for assaying progesterone. The limitations of each are discussed.

2. Attention is called to the value and uses to which absorption spectrophotometry may be put in this respect. The merits and difficulties of making determinations in the infra-red and ultraviolet regions of the spectrum are discussed.

3. Absorption curves for a number of steroids were determined, and found to agree with those in the literature. The fact is brought forth that, by using selective chemical procedures, one may obtain in one fraction the neutral, non-volatile compounds of high molecular weight, with appropriate chromogenic activity for progesterone limited to the ketonic fraction along with estrogens.

4. The characteristics of progesterone, and estrogen, absorption curves are such that the two hormones may be measured simultaneously.

5. The test for progesterone is characteristic for alpha, beta unsaturated ketones; by employing suitable chemical procedures, one may be reasonably certain that he is dealing with Δ^4-3 ketosteroids.

6. The method does not distinguish between testosterone, corticosterone and related substances, androstenedione and progesterone.

7. As developed at present, the method is limited to those experimental conditions in which it is certain or highly probable that he is dealing with progesterone, or one of the other specific, Δ^4-3 ketosteroids.

8. The limitations, sensitivity and details for improvement are outlined.

9. The type of problem to which the method may be used logically is indicated.

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A SPAYED-RAT ESTROGEN ASSAY METHOD AND ITS APPLICATION TO PURE ESTROGENS AND URINE EXTRACT[1]

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WITH THE ASSISTANCE OF DONALD OTWAY

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THE METHOD described here was devised for the assay of the phenol fraction from urine extracts in which the concentration of estrogenic material was too low to assay by the usual spayed rat method (1–4). Preliminary studies indicated that a weekly priming with pure theelin approximately 48 hours before injecting the unknown made the rats much more sensitive. Subsequent studies and the application of the method as finally adopted confirm this and the data presented here establish the reliability of the method which has been in use in this laboratory for the past five years.

The estrogen preparations and standards used in these studies were made up to definite concentrations in sesame oil by the author and submitted to Mr. Donald Otway for assay under a code unknown to him. The standards were prepared by dissolving pure, crystalline theelin[2] in sesame oil.

The following procedure was adopted. Albino rats from our own colony were spayed at 3 months of age. One to two months later they are available for preliminary assays. Each rat is injected subcutaneously in the back through a #22 needle 2 inches long, with 0.1 cc. of a sesame oil solution containing 1 microgram theelin. This is the priming dose.

Forty-eight hours later the rats are injected subcutaneously in the same way with 0.25 cc. of the sesame oil solutions of standards and unknowns. At least two levels, 0.5 and 1.0 μg. of pure theelin, respectively, are used. In special cases other standards of 0.25 and 0.75 μg. may be included. Twenty rats are used for each unknown and standard.

Vaginal smears with a cotton swab, moistened with normal saline are made 48 hours later. The rats which do not show an estrous smear (*E* or cornified epithelial cells without leucocytes) are smeared again 4 hours later. The smears are read, without staining, immediately after the drop has been placed on the slide and each is recorded as *L*, *LE*, *EL*, *EEL*, or *E* depending on the preponderance of leucocytes or epithelial cells. The rats can be used in the same way every week for 4 to 5 months. They are, of course, primed in the same way each week.

In evaluating the responses the percentage of *E*, *EEL* and *L* on standards and unknowns are compared and the latter expressed as μg. equivalents of theelin.

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[2] The theelin was supplied by Dr. George Cartland of the Upjohn Co., Kalamazoo, Mich. The other pure estrogens were supplied by Dr. O. Wintersteiner of E. R. Squibb & Sons, New Brunswick, N. J., and by Dr. E. Schwenk of the Schering Corp., Bloomfield, N. J.

Standards must be run each week in parallel with unknowns in order to eliminate the variations observed from week to week.

The rats usually are not sufficiently sensitive to respond to 0.5 μg. of theelin when first used. Hence it is desirable to use all of the rats each week for at least 3 weeks on known doses, 0.25 to 1.0 μg. of theelin, before using them for regular assays. Readings under 5 or over 70 per cent *E* are not of much value.

EXPERIMENTAL OBSERVATIONS

In the first critical experiment 200 rats were followed for 10 consecutive weeks on 0, 0.25, 0.50, 0.75, and 1.0 μg. of theelin with 20 rats at each level each week. In

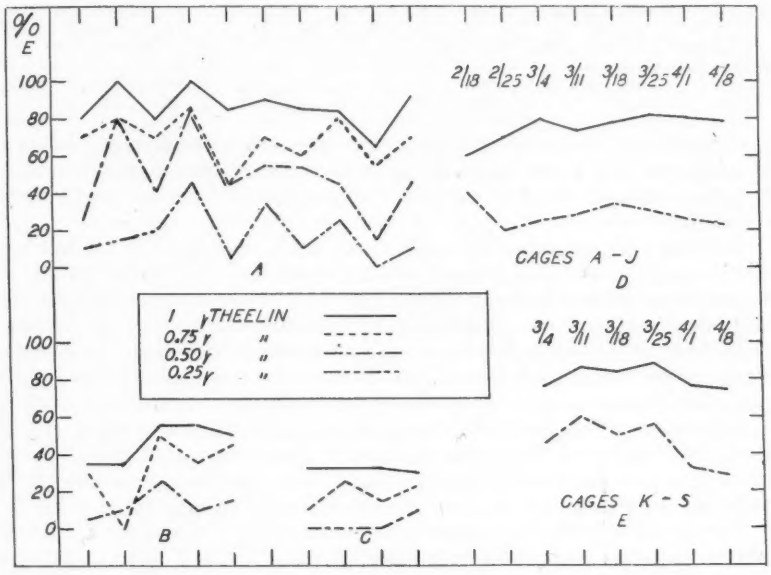

Fig. 1. Weekly variations in percentage of estrous response to theelin.

this experiment they were primed with 1 μg. of theelin dissolved in 0.25 cc. of sesame oil, and 48 hours later the unknowns were injected and the assay completed as previously described. Figure 1 (*A*) shows the week to week variations on each level. The grand average percentage *E* values for 1, 0.75, 0.50, 0.25, and 0 μg. of theelin were 86, 69, 49, 19, and 0.5 per cent, respectively. It is obvious that there are distinct weekly fluctuations, but the tendency is for all of the rats either to decrease or to increase in sensitivity from one week to another. There is no evidence of an accumulative effect.

In the second experiment the rats were primed each week with 0.75 μg. of theelin in 0.1 cc. of sesame oil. The unknowns were injected as usual 48 hours after the priming. Smears made 52 hours after the priming dose did not give any *E* smears, but the smears made 48 and 52 hours after the unknowns gave average percentage *E* values of 26, 5.5, 8.5, 2.5 and 0.5 for 1, 0.66, 0.53, 0.33 and 0 μg. of theelin for 10 consecutive weeks. A repetition of this experiment on the same priming dose, but without the

TABLE 1. RESULTS OF ASSAYS OF THEELIN IN SPAYED RATS USED IN PREVIOUS ASSAYS

Date	2/18/41		2/25/41		3/4/41		3/11/41		3/18/41		3/25/41		4/1/41		4/8/41	
Rats	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %
A	0	0	0	0	0	0	1.0	85	1	75	1	95	1	80	1	90
B	0	0	0	0	0	0	1.0	60	1	80	1	90	1	75	1	85
C	0	0	0	0	0	0	0.5	35	0.5	35	0.5	25	0.5	15	0.5	15
D	0	0	0	0	0.5	25	0.5	35	0.5	40	0.5	25	0.5	35	0.5	25
E	0	0	0	0	1.0	75	0.5	25	1.0	75	0.5	40	1.0	80	0.5	25
F	0	0	0	0	0	0	0.5	20	1.0	90	0.5	25	1.0	85	0.5	25
G	0	0	1.0	70	0	0	1.0	65	0.5	30	1.0	70	0.5	20	1.0	80
H	0	0	0.5	20	0	0	1.0	75	0.5	35	1.0	85	0.5	30	1.0	75
I	0.5	40	0	0	0	0	0.5	25	0.5	30	1.0	70	0.5	25	1.0	65
J	1.0	60	0	0	0	0	1.0	85	1.0	70	0.5	45	not used		not used	
Average values	0.5	40		20		25		28		34		30		25		23
	1.0	60		70		75		74		78		82		80		79

vaginal smear 52 hours after the priming dose, gave average percentage *E* values of 46, 32, 15, and 0 for 1, 0.75, 0.50 and 0 μg. of theelin. The weekly fluctuations shown in figure 1 (*B*) again illustrate the same tendency toward a uniform weekly trend in sensitivity.

In figure 1 (*C*) are shown the results from a similar experiment, but in this case the priming dose was 0.90 μg. of theelin and 40 rats were used on each level each week. The responses resulted in low percentage *E* values and were rather irregular. On 0.25 and 0 μg. of theelin the vaginal smears were uniformly negative in the *E* response.

In the experiments described thus far no attempt was made to control the selection of rats for the various levels of estrogens. The observations listed in tables 1 and 2 were made in order to determine to what extent a previous week's treatment affects the vaginal smear response. In this series the animals were primed with 1 μg. of theelin in 0.1 cc. of oil. The unknowns were injected as 0.25 cc. samples. Twenty rats were in each set. *Rats A* to *J* inclusive had been used for routine assays for 4 weeks and *rats K* to *S*, inclusive, for 8 weeks prior to starting this experiment.

The results in tables 1 and 2 show that a previous week's treatment such as indicated in the tables does not seriously affect the percentage of *E* response the next week. Unfortunately the high percentage *E* response on 1 μg. in these rats makes the

TABLE 2. RESULTS OF ASSAYS OF THEELIN IN SPAYED RATS USED IN PREVIOUS ASSAYS

Date	3/4/41		3/11/41		3/18/41		3/25/41		4/1/41		4/8/41	
Rats	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %	Dose μg.	E %
K	1	75	1	90	1	85	1	80	1	85	0.5	20
L	1	70	1	75	1	80	1	65	1	80	0.5	25
M	1	80	1	95	1	90	1	80	1	65	0.5	30
N	1	70	1	85	1	85	1	70	1	65	0.5	30
O	1	85	0.5	60	1	80	0.5	55	1	85	0.5	35
P	0.5	40	1	85	0.5	55	1	75	0.5	35	1.0	75
Q	0.5	50	1	90	0.5	50	1	75	0.5	20	1	80
R	0.5	35	1	85	0.5	40	1	85	0.5	40	1	85
S	0.5	55	1	90	0.5	50	1	75	0.5	35	1	65
Average values	0.5	45		60		49		55		32		28
	1.0	76		87		84		88		76		75

TABLE 3. ASSAYS FOR THEELIN IN URINE IN SPAYED RATS

Cc. of Stock Urine Extract in 6 cc. Sesame Oil	Relative Doses μg.	Number Rats	% Estrous Response
4.60	1.00	20	55
3.45	0.75	20	35
2.30	0.50	20	25
1.15	0.25	20	15

observation at that level of doubtful value, but it is perfectly clear that the response to 0.5 μg. of theelin from week to week is not affected by the previous weeks' treatments. A synchronous rise and fall for the average percentage of *E* responses on 0.5 and 1.0 μg. is again shown each week for both series of rats (fig. 1, *D* and 1, *E*).

Rats A to *J* inclusive were next used for routine assays for 2 weeks and on the third week after the experimental series were primed as usual. All of the vaginal smears made 96 and 100 hours after the priming dose were negative. This shows that the priming dose alone does not produce a positive vaginal smear at the usual time of smearing for unknowns. A week later, when the animals were used for regular assays, they gave percentage *E* values of 85; 45, 50 and 60; 25 and 40, and 10 and 15 on 1, 0.75, 0.50 and 0.25 μg. of theelin, respectively. Very similar observations were made on *rats K* to *S* after two weeks' routine assays on urine concentrates.

Table 3 shows that the method gives very satisfactory results on different doses of urine extracts, although the actual slope of the curve may not be the same in all urines, or with various concentrations of pure estrogens.

Table 4 gives the comparative estrogenic values of pure estrogens by this method. Very irregular results were obtained with estriol and various esters of the estradiols. With the esters it was very difficult to determine the minimum dose which caused a 25 to 60 per cent *E* response in 48 to 52 hours after injection. When doses of 5 to 20 μg. of the ester forms were used some of the rats remained in estrus for at least 30 days. This was especially true for estradiol dipropionate. Obviously the colony cannot be used every week when ester forms have been injected. Comparison of the imma-

TABLE 4. COMPARATIVE ESTROGENIC VALUES OF PURE ESTROGENS

Substance	Relative Activities by:	
	Spayed rat method	Immature mouse-uterine weight method (4)
Estrone	100	100
α-Estradiol	1000	300
β-Estradiol	10 very irregular	7.5
Estriol	<20 very irregular	40
7 keto-estrone	3	7.5
Δ6-Equilin	<25	7.5
β-dihydroequilenin	8 approximately	6
Equilin	25 approximately	110
Diethylstilbestrol	150–200 irregular	250
α-Estradiol benzoate	<25 very irregular, but prolonged action	200
α-Estradiol dipropionate	very irregular, but prolonged action	70
α-Estradiol diacetate	<10 very irregular, but prolonged action	110
β-Estradiol benzoate	<3	10
β-Estradiol acetate	<2	0.1
β-Estradiol, 3 benzoate	12–24	20
Progesterone	— 0 at 100 μg.	0.2

ture mouse uterine weight response with the present method shows a striking lack of quantitative parallelism.

SUMMARY AND CONCLUSIONS

1. Priming weekly with 1 μg. of theelin in 0.1 cc. of sesame oil, followed by an injection of 0.25 cc. of sesame oil solution of standards and unknowns containing up to 1 μg. of theelin or the equivalent gives very reliable vaginal smear responses 48 and 52 hours after the injection of the standards and unknowns.

2. Direct reading of smears without staining is reliable and less time consuming.

3. There is no evidence that, in the weekly use of spayed rats by this method, there is an accumulative estrogenic effect. It is true that in the first 2 to 4 weeks' use after spaying one finds the rats less sensitive, but after that the sensitivity in the colony fluctuates from week to week. It is very probable that the smearing makes them somewhat more sensitive.

4. The method was first devised to assay urinary preparations very low in estrogens; hence the weekly priming. It is easily possible that weekly priming is not necessary if ample estrogenic material is available for the assay. This should be studied.

5. The method has been used very successfully in routine assays on human urines, in the assay of commercial preparations, in comparative studies on numerous pure estrogens and in inhibition studies on androgens.

6. When certain esters of α-estradiol are assayed by this method the results are very irregular. Doses of these esters which cause a 50 per cent estrous smear in 48 to 52 hours after injection cause prolonged estrus for weeks, depending on the dose.

7. The prolonged action of such esters obviously makes such animals of no value for weekly assays, until properly standardized.

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QUANTITATIVE STUDY OF ANTAGONISM OF ESTROGEN AND PROGESTERONE IN CASTRATE RABBIT[1]

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IN A PREVIOUS communication (1) we reported on the quantitative inhibition of estradiol benzoate by progesterone in the immature and adult castrate baboon (*Papio porcarius*). The criteria used for this purpose were based on the perineal deturgescence and the onset of progesterone-withdrawal bleeding. No proportional relationship was found in the antagonism of estradiol benzoate and progesterone such as was said to exist in other animals. Instead we discovered that an adequate or optimal amount of progesterone could antagonize effectively large quantities of estradiol.

The antagonism of these hormones has been examined in the mouse (2), in the rat (3), in the rabbit (3, 4, 5), in the monkey (6) and in the Algerian monkey (3). The rabbit has been the animal commonly employed for this purpose, but Robson (2) alone has presented a detailed quantitative analysis of the antagonism between estrogen and progesterone. From a careful study he concluded that the ratio of the two hormones when used simultaneously was the essential factor in determining the degree of progestational response induced in the uterus.

In view of the fact that in the baboon estradiol benzoate was not antagonized quantitatively by progesterone, it seemed desirable to ascertain whether the rabbit was different from the baboon in this respect.

MATERIAL AND METHOD

The technique adopted throughout these experiments was similar in all respects to that employed by Robson (2). Adult female rabbits were ovariectomized and 14 days later were subjected to experimental procedures.

In our earlier experiments only estradiol benzoate was available, but later when we obtained estrone we were able to duplicate and extend the various experiments conducted by Robson. Estrogen was administered daily for 7 days making a total of 0.00588 mg. of estradiol benzoate or 0.07 mg. of estrone. The amount of estradiol benzoate was based arbitrarily on Astwood's findings in the rat, namely, that estradiol benzoate is 12 times more potent than estrone (7). We did not test this out in the rabbit as it is exceedingly difficult to estimate the end-point of optimum estrogen activity in this animal. After this preliminary treatment with estrogen, estrogen and progesterone were injected simultaneously in 8 divided doses for 4 days, and the animal was autopsied a day later.

In some of our control experiments progesterone alone was used with and without preliminary sensitization with estrogen.

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[1] This investigation was aided by a grant from the South African Research Council and Board.

TABLE 1. EFFECT OF PROGESTERONE ALONE, WITHOUT PREVIOUS ESTROGEN SENSITIZATION, ON THE PROGESTATIONAL REACTION

Experiment Number	Progesterone, mg.	Progestational Reaction
1	0.75	0
2	0.75	0
3	0.8	0
4	1.5	+
5	1.6	++
6	7.0	+
7	7.0	±
8	20.0	+

In order to be able to standardize the degree of progestational reaction in the rabbit uterus, we used for comparison samples of typical positive and negative reactions generously donated to us by Dr. Robson.

EXPERIMENTAL RESULTS

Effect of progesterone alone. The results of treatment of castrated rabbits with progesterone alone without previous estrogen sensitization are shown in table 1. It is evident that a progestational reaction was not evoked with less than 1.5 mg. of progesterone. With 1.5 mg. a 1 or 2 plus reaction was elicited but larger amounts up to 20 mg. did not intensify the response. This latter finding was contrary to expectation and is noteworthy.

Effect of progesterone after estrogen sensitization. After 7 days of treatment with a total of 0.07 mg. of estrone or 0.00588 mg. of estradiol, followed by 4 days of treatment with progesterone alone it was found that the effectiveness of small quantities of progesterone was noticeably enhanced. Thus without pretreatment with estrogen, 0.75 mg. of progesterone did not produce a positive response, whereas after estrogen pretreatment this amount of progesterone caused a 2 plus progestational response which was only possible with 1.5 mg. of progesterone without estrogen sensitization (cf. tables 1 and 2).

Effect of simultaneous administration of estrogen and progesterone after estrogen sensitization. Since 0.75 and 1.5 mg. of progesterone alone after estrogen sensitization give a marked progestational reaction (table 2), it was decided to maintain the progesterone at these levels in the first series of combined estrogen and progesterone experiments. The results with estradiol benzoate are shown in table 3, A, and with estrone in table 3, B.

A combination of 0.75 mg. of progesterone and 0.0025 mg. of estradiol benzoate (ratio 300:1) resulted in a 2 plus progestational reaction. With increasing amounts of estradiol and a corresponding decrease in the progesterone:estradiol ratio, the reaction became doubtful or even absent. With 1.5 mg. of progesterone and estradiol benzoate of less than 0.005 mg. (ratio 300:1 and higher) the progestational reaction

TABLE 2. EFFECT OF PROGESTERONE ALONE AFTER PRELIMINARY ESTROGEN SENSITIZATION

Experiment Number	Preliminary Estrogen,[1] mg.	Progesterone, mg.	Progestational Reaction
9	0.00588	0.75	++
10	0.00588	1.50	+++
11	0.07	0.75	++

[1] Experiment 9 and 10, estradiol benzoate; experiment 11, estrone.

TABLE 3,A. EFFECT OF VARYING QUANTITIES OF ESTRADIOL BENZOATE COMBINED WITH A FIXED AMOUNT OF PROGESTERONE

Experiment Number[1]	Estradiol Benzoate, mg.	Progesterone, mg.	Progestational Reaction	Progesterone: Estradiol Ratio
12	0.01	0.75	±	75:1
13	0.005	0.75	±	150:1
14	0.0025	0.75	++	300:1
15	0.02	0.75	±	37.5:1
16	0.1	0.75	0	7.5:1
17	0.01	1.5	+	150:1
18	0.005	1.5	++	300:1
19	0.0025	1.5	++	600:1
20	0.04	1.5	±	37.5:1

[1] All animals sensitized with 0.00588 mg. estradiol.

TABLE 3,B. EFFECT OF VARYING QUANTITIES OF ESTRONE COMBINED WITH A FIXED AMOUNT OF PROGESTERONE

Experiment Number[1]	Estrone, mg.	Progesterone, mg.	Progestational Reaction	Progesterone: Estrone Ratio
21	0.2	0.75	0	3.75:1
22	0.01	0.75	0	75:1
23	0.08	1.5	0	18.5:1
24	0.04	1.5	0	37.5:1
25	0.02	1.5	+	75:1
26	0.0025	1.5	+++	600:1

[1] All animals sensitized with 0.07 mg. estrone.

was well marked; with increasing amounts of estradiol and a corresponding diminution in the progesterone:estradiol ratio the reaction was less or doubtful.

Similar results were obtained with estrone. With 1.5 mg. of progesterone and 0.0025 mg. estrone (ratio 600:1) a well-marked progestational reaction was elicited, whereas 0.02 mg. of estrone with the same quantity of progesterone (ratio 75:1) produced a weaker response. With still larger quantities of estrone the reaction was negative.

These results, at first sight, seemed to suggest that Robson was correct in stating that progesterone was quantitatively inhibited by estrogen, and that the presence of a progestational reaction was dependent on the quantitative ratio of these two hormones. In these experiments a ratio of progesterone to estrogen of 150:1 or higher produces a progestational reaction.

TABLE 4. EFFECT OF A COMBINATION OF PROGESTERONE AND ESTROGEN IN RATIOS OF 150:1 AND OVER, USING LARGE AMOUNTS OF THE HORMONES

Experiment Number[1]	Estrogen (mg.)	Progesterone (mg.)	Progestational Reaction	Progesterone: Estrogen Ratio
27	0.004	8.0	+	2000:1
28	0.04	8.0	±	200:1
29	0.08	16.0	0	200:1
30	0.01	3.0	±	300:1
31	0.02	6.0	±	300:1
32	0.04	12.0	+	300:1
33	0.08	12.0	0	150:1

[1] Estradiol benzoate used in experiments 27–29; estrone in 30–33.

In order to test the validity of this thesis we conducted additional experiments in which larger amounts of progesterone and estrogen were used, and in which the ratio was maintained at a level of 150:1 and higher. The results are shown in table 4. It is at once apparent that, when the high ratio is maintained with substantial increase in the dosage of estrogen and progesterone, the progestational reaction is less than that obtained with lesser amounts of these hormones even though the ratios are similar. This indicates therefore that the ratio of estrogen and progesterone is not the sole factor in determining the progestational reaction, but that the response is significantly influenced by the absolute amounts of these hormones.

DISCUSSION

Robson (5) investigated the quantitative inhibition of estrone by progesterone in the rabbit endometrium and indicated that this appeared to obey a mathematical law of direct proportional variation. He used chiefly 0.75 and 1.5 mg. of progesterone with varying amounts of estrone, and he concluded that when the progesterone:estrone ratio was above 75:1 a progestational reaction would be obtained. Unfortunately he did not experiment with larger amounts of estrone and progesterone.

Our experiments with 0.75 and 1.5 mg. of progesterone in essence confirm the results of Robson. When, however, larger quantities of estrogen and progesterone are used, the progestational reaction is inhibited in spite of maintaining the estrogen: progesterone ratio which was effective with 0.75 and 1.5 mg. progesterone. Robson's conclusion that a true quantitative antagonism of these substances exists is based on the fortuitous use of optimal quantities of progesterone and estrogen. When the optimal range is exceeded the ratio of the two hormones no longer obtains as the only factor determining progestational response.

Our experiments might be criticized on the grounds that we have relied chiefly on estradiol benzoate, whereas Robson used estrone. The preliminary sensitization used has been equivalent on the basis that estradiol benzoate is regarded by Astwood (7), from his observations on the rat, as being approximately 12 times as potent as estrone. The subsequent use of estradiol and estrone in conjunction with progesterone has not been corrected in estimating the progesterone:estrogen ratio. Thus a ratio of progesterone:estradiol benzoate of 600:1 might be regarded as equivalent to a ratio of progesterone:estrone of 50:1. This would more seriously have questioned the validity of the quantitative inhibition of estrogen and progesterone, since progesterone:estradiol benzoate ratios of 6:1 and 12:1 would have given good progestational reactions. The introduction of such a correction factor, however, would only have served to complicate the issue. Optimal preliminary sensitization is an indispensable requisite for the progestational reaction; thereafter, irrespective of whether estradiol benzoate or estrone is combined with progesterone, provided that the doses are within the optimal range, the response will be positive. The optimal range of estrogen apparently lies below the original sensitizing dose. When the amount of estrogen approximates the original sensitizing level, and even exceeds it, the progestational reaction becomes less marked or inhibited. With this elevated level of estrogen proportionately large quantities of progesterone do not induce an effective progestational response.

The rabbit appears to be more sensitive to estrogen than to progesterone. Estrogen above a certain limit appears to prevent an extraordinarily large quantity of progesterone from exerting its progestational effect. In the baboon, on the other hand, an optimal dose of progesterone could successfully antagonize excessive amounts of estradiol benzoate. In a previous communication (8) we suggested the hypothesis that

in the evolutionary scale, the more highly developed animals became more sensitive to progesterone. Although it is difficult to compare the estrogen-progesterone inhibition in the rabbit and the baboon, certain similarities may be noted. In both, the quantitative inhibition is not dependent on a mathematical law of direct proportional variation of the two hormones. In both, optimal amounts of the hormones must be present to induce the desired response (perineal deturgescence and progesterone-withdrawal bleeding in the baboon, and progestational proliferation in the rabbit). However, in the rabbit estrogen antagonizes large quantities of progesterone, whereas in the baboon progesterone antagonizes large quantities of estrogen.

Summarizing our experiments on more than 70 castrate rabbits, three reactions may be observed. *a*), With optimal amounts of both hormones a progestational response is elicited; *b*), with larger amounts of both hormones, when used in a ratio which was effective in producing a progestational reaction in the optimal range, the progestational response is reduced or inhibited; *c*), with excessive amounts of estrogen and progesterone, intense vascular reactions are produced (9).

SUMMARY

The quantitative antagonism of estrogen (estradiol benzoate and estrone) and progesterone has been examined in the castrate rabbit, using the degree of progestational proliferation of the endometrium as an indicator.

A quantitative ratio is present when optimal amounts of estrogen and progesterone are used, and the experiments of Robson (8) are confirmed. When estrogen is increased, the progestational reaction is diminished or inhibited. When large amounts of both hormones are used in a ratio which was effective in producing a progestational reaction in the optimal range, the progestational response is reduced or inhibited. The progestational proliferation is dependent on the presence of optimal amounts of estrogen and progesterone, and cannot be represented as following a mathematical law of direct proportional variation.

We wish to express our appreciation to Prof. Raymond A. Dart in whose Department this investigation was pursued, for his advice and criticism, and to Prof. G. Buchanan for his interest and encouragement. We especially wish to acknowledge our indebtedness to Miss C. Gilbert for her invaluable assistance with the technical aspects of this investigation. We are also particularly indebted to Dr. J. M. Robson of Edinburgh who so kindly sent us samples of histological specimens of rabbit uteri used by him in his own study.

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A QUANTITATIVE STUDY OF THE BLEEDING THRESHOLD AND OPTIMUM ESTROGEN REQUIREMENTS IN THE BABOON (*PAPIO PORCARIUS*)[1]

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ALTHOUGH the estrogen bleeding threshold has been established for monkeys (1, 2), no data are available concerning the minimum amount of estrogen required to produce an effect in the genital system identical with that induced by endogenous estrogen elaborated by the monkey's own ovaries during the first part of the cycle.

We are not entitled to claim that the minimum amount of estrogen necessary to cause bleeding is identical with that secreted by the animal from the time of menstruation up to ovulation, even though there is a marked similarity in the histological appearances presented by these two types of endometrium at the height of estrogen effectiveness. With ordinary histological techniques the structure of two specimens of endometrium may appear to be the same, and yet with more sensitive histochemical procedures a very distinct difference may become manifest. This has been clearly shown in the human endometrium (3, 4, 5). If we are to obtain a true reflection of the rôle of estrogen in the first part of the cycle it is of paramount importance that a relationship (if any) be established between the minimum amount of estrogen necessary to cause estrogen-withdrawal bleeding and optimum estrogen effectiveness.

The reason for the lack of this data concerning the optimum estrogen effectiveness is no doubt due in part to the fact that there is not available an easy and reliable technique for this purpose. The technique for determining the estrogen-bleeding threshold is relatively simple. The onset of bleeding, the criterion invariably employed, usually provides a sharp end point. Experiments may be repeated on the same animal without subjecting the animal to a corresponding number of surgical procedures which would become indispensable in studies to ascertain the estrogen requirements for optimum estrogen action. As already pointed out above even biopsies of the endometrium would not necessarily be very helpful.

The baboon, however, is ideal for such studies, for not only does it menstruate regularly but it also possesses a sexual skin which reflects accurately the day-to-day effect of estrogen. The perineum has already proved its reliability in studies involving the use of estrogen and progestogen (6, 7, 8).

In studies on optimum estrogen effectiveness it is especially valuable in that it is possible to establish in the intact animal the fluctuations in the size of the perineum over a period of a large number of cycles and then such an animal may be castrated. Thereafter it is possible by means of estrogen to reproduce the same type of perineal response as occurred normally under the influence of endogenous estrogen.

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The object of this paper is *a*), to establish the minimum amount of estrogen required to produce withdrawal bleeding in baboons; *b*), to ascertain the amount of estrogen necessary to cause the earliest visible perineal response; *c*), to determine the minimum amount of estrogen required to produce an optimum perineal response such as is seen in the normal baboon toward the end of the first half of the cycle. Finally, a quantitative comparison will be made of the minimum amount of estrogen found necessary to excite estrogen-withdrawal bleeding with the corresponding amount of estrogen required to evoke a minimal and an optimal perineal response. From this data it will be shown that, in the majority of animals used in this investigation, there is a difference between the estrogen-bleeding threshold values and optimum estrogen activity as assessed by the perineal swelling.

MATERIAL AND METHODS

In experiments of this character the normal menstrual rhythm of adult female baboons has been observed for a sufficient length of time to be certain within experimental error, not only of the length of the menstrual cycle, but also of the fluctuation in the size of the perineum. It has been pointed out in an earlier study (9) that female baboons brought into captivity go into anestrus for a variable period up to 9 months. Only when they become adjusted to their new environment do they commence to menstruate normally. For this reason the menstrual data of 10 adult female baboons used in this study has been recorded for a period of not less than 3 years. Only after data from a sufficient number of cycles of each baboon were available, in order to know the normal reactions of the perineum of each baboon, were the animals castrated. When the perineal swelling had become completely absorbed, such as occurs normally in prepubertal baboons, then only was treatment with estrogen started.

The estrogen used was in the form of crystalline estradiol benzoate dissolved in sesame oil so that each dose was contained in not more than 0.2 cc. of the oil. Unfortunately at the time these experiments were started estrone was not available to me so that it is not possible to compare directly the findings in baboons with those in the macaque. Now that estrone may be obtained it is hoped at a later date to examine the relative effectiveness of estradiol benzoate and estrone; at the same time this data will allow of a comparison with those excellent data already established for the monkey (2, 3, 13).

The animals were injected daily for 15 days with the requisite amount of hormone. The same amount of estradiol benzoate was injected daily. In the baboons used in this study the perineum usually became maximally turgescent before and certainly not later than the 15th day of the cycle. A week after the onset of withdrawal bleeding a new experiment was started. As a rule the same experiment was repeated 3 and sometimes 4 times on the same animal for reasons to be mentioned later. Over 120 experiments were performed on the 10 baboons. In each instance daily measurements were made of the size of the perineum.

No mention will be made of the histological appearance of the endometrium during these experiments as a sufficient number of normal uteri in the first part of the cycle are not yet available for comparison.

EXPERIMENTAL RESULTS

The bleeding threshold. The results of the findings are summarized in table 1. Before examining table 1 it is necessary to mention that 3 animals (*B23*, *B45* and *B43*) would not bleed when subjected to the same amount of estradiol benzoate which previously was able to excite withdrawal bleeding. In such animals the threshold

value was only regarded as having been established when withdrawal bleeding was produced consistently in a series of 3 experiments with the same amount of estradiol.

From the data in table 1 it is evident that of the 10 animals 7 bled consistently after a minimum daily amount of 0.01 mg. of estradiol. Of the remaining 3, 1 bled after 0.02 mg., 1 after 0.03 and one (*B42*), although it bled once with as little as 0.01 and 0.02 mg., nevertheless on 2 consecutive occasions did not bleed after a daily dose of 0.04 mg. This animal, as compared with other animals, is extremely erratic and is resistant to estradiol benzoate.

Nine animals bled at least once after daily treatment with 0.01 mg. of estradiol, and every animal bled at least once after 0.02 mg. *B23* bled once in 3 experiments after 0.01 mg. but bled in every instance of the 4 experiments in which 0.02 mg. of estradiol was injected daily. *B45* bled 2 of 3 times after 0.02 mg. but bled consistently in 4 experiments involving the use of 0.03 mg. of estradiol. *B43* bled once in 3 experiments with 0.01 mg., once in 2 with 0.02 mg. and once in 4 times after the use of 0.04 mg. of estradiol.

TABLE 1. RELATIONSHIP BETWEEN DAILY AMOUNT OF ESTRADIOL BENZOATE NECESSARY TO CAUSE ESTROGEN WITHDRAWAL BLEEDING AND MINIMAL AND OPTIMAL PERINEAL TURGESCENCE

Baboon Number	Minimum Amount of Estradiol to Cause Bleeding	Minimum Amount of Estradiol to Cause Minimal Visible Perineal Reaction	Minimum Amount of Estradiol to Cause Optimal Perineal Reaction
	mg.	*mg.*	*mg.*
15	0.01	0.01	0.04
16	0.01	0.01	0.04
22	0.02	0.01	0.04
23	0.01	0.01	0.01
30	0.02	0.01	0.03
31	0.01	0.01	0.02
36	0.02	0.02	0.04
43	0.06	0.01	0.04
45	0.03	0.01	0.03
60	0.01	0.01	0.02

It can be seen from table 1 then that in 5 animals the minimum daily requirements of estradiol benzoate given for 15 days to cause withdrawal bleeding was 0.01 mg., in 3 it was 0.02 mg., in 1, 0.03 mg., and in 1, 0.06 mg.

Minimum amount of estradiol benzoate necessary to cause a visible change in the perineum. In the castrate baboon the perineum reverts to a condition which is similar to that seen in a prepubertal animal. The sexual swelling is completely absorbed and there is a marked depression between the anus and vagina medially and the ischial callosities laterally. The earliest evidence of the reaction of the perineum to estrogen manifests itself in the form of a slight peri-anal swelling which rapidly spreads laterally towards the callosities, as well as downwards to involve the clitoris.

In 3 animals a very slight perineal swelling was noticed after 0.005 mg. of estradiol. In 9 animals there was a positive reaction after 0.01 mg. (table 1) and the 10th responded to 0.02 mg.

Minimum amount of estradiol benzoate necessary to cause an optimal swelling of the perineum. The maximum dimensions of the perineum were established before castration in all the 10 baboons. The maximum size of the swelling at the height of estrus varies for each individual animal. The size aimed at in these experiments was the minimum size which the animal normally attained just before the onset of perineal

deturgescence. The perineum responds in a quantitative fashion to different concentrations of estradiol benzoate. This is more marked in some animals than in others, depending largely on the size of the daily injection necessary to cause optimum perineal turgescence. When the daily requirements of estradiol benzoate are relatively large (0.04 mg.) then it is possible to appreciate the effects of smaller doses on the increments in size of the perineum. This is clearly shown in table 2.

TABLE 2. EFFECT OF INCREASING AMOUNTS OF ESTRADIOL BENZOATE ON SIZE OF PERINEAL SWELLING IN CASTRATE BABOONS

Daily Amount of Estradiol, mg.	Maximum Width of Perineum in Inches				
	B15	*B16*	*B22*	*B36*	*B43*
0.0025					
0.0050		4.00	6.00		
0.0100	4.50	5.75	4.50	p-a-s[1]	5.25
0.0200	5.50	7.00	7.00	5.5	7.50
0.0300	6.50	8.00	7.50	7.0	7.75
0.0400	7.25	9.00	8.75	8.5	9.00
Maximum size of perineum before castration	7	9	9	9	8.75

[1] Peri-anal swelling.

By using the perineal skin as an indicator of optimum estrogen metabolism it was discovered that in 1 animal the perineum reached a size comparable with that produced by its own ovaries after daily treatment with 0.01 mg. of estradiol, 2 animals required 0.02 mg., 2 animals required 0.03 and 5 animals required as much as 0.04 mg. per diem.

DISCUSSION

Bleeding threshold. Although at first sight there seems to be considerable variation in the amount of estradiol necessary to cause withdrawal bleeding in baboons, nevertheless from table 1 it is evident that in 8 of the 10 animals the threshold value for bleeding was between 0.01 and 0.02 mg. of estradiol benzoate. No animal bled after a daily amount of 0.005 mg.

Although it is not possible to compare the findings in the baboon with those in the Macacus rhesus it appears that in the macaque the overwhelming majority of the animals used by Zuckerman bled after daily treatment with 50 to 100 I.U. of estrone, but as in the baboon, some monkeys required less and others more.

It must be accepted that in the baboon as in the monkey, animals have different degrees of sensitivity to estrogen. In normal women too, there is a great individual variation in sensitivity not only to estrogen but also to progesterone (10, 11, 12).

Apart from this individual sensitivity to estrogen Zuckerman (2) drew attention to another phenomenon in monkeys, namely, a rhythmical elevation of the estrogen threshold in the same animal.

At one stage during these experiments on baboons it was thought that a similar phenomenon existed in baboons, but apparently it is not quite the same. For example *B23* received 0.01 mg. of estradiol daily for 15 days and 13 days after the last injection, withdrawal bleeding supervened. This animal was treated on two subsequent occasions in the same way and yet no withdrawal bleeding occurred. With higher concentration of the hormone, withdrawal bleeding was a regular feature. *B43* reacted in the same manner as *B23*, but in addition it was found that on 3 occasions no bleed-

ing occurred after daily treatment with as much as 0.04 mg. of estradiol, although it bled previously after daily amounts of 0.02 mg. and 0.03 mg. of estradiol.

Whatever the nature of these aberrant reactions, any consideration of them falls strictly outside the scope of this paper. Zuckerman (13) was of the opinion that fluctuations in the level of the threshold values for estrogen were due to cyclical changes in the adrenals. These and other adrenal problems are receiving our attention (14).

In general, however, it may be stated that despite the existence of individual sensitivity, and with the possible exception of *B43*, the threshold for estrogen-withdrawal bleeding in baboons is fairly constant ranging between 0.01 and 0.02 mg. of estradiol benzoate per day.

Quantitative relationship between the bleeding threshold and the perineal reactions. Although 3 animals responded, at least on one occasion, by showing a slight turgescence of the perineum after daily treatment with 0.005 mg. of estradiol, a very slight but consistent perineal response was elicited in 9 of 10 animals with daily amounts of 0.01 mg. of estradiol benzoate. From table 1 it appears that slightly less estradiol benzoate is necessary to cause a visible, albeit a slight, perineal reaction than is necessary to cause estrogen-withdrawal bleeding. The perineal skin, therefore, is very sensitive to estrogen.

The amount of estrogen necessary to cause an optimum perineal response in the castrate, such as was normally encountered at about the time of ovulation in the uncastrated baboon, is much greater than the estrogen requirement for a minimum reaction; in 4 of 10 baboons it was at least double.

If we compare the minimum estrogen requirements to cause withdrawal bleeding and optimal perineal response we see from table 1 that with one exception (*B43*), the estrogen requirements for the optimal perineal response are greater than for bleeding; it was the same in 2 animals (*B23*, *B45*); it was twice as much in 4 animals (*B22*, *B31*, *B36*, *B60*), it was 4 times as much in 2 animals (*B15*, *B16*) and in 2 animals the two levels were identical.

The perineal swelling is obviously the better indicator of estrogen requirement than bleeding. Furthermore, if in a mature animal under the influence of its own estrogen, the perineum reaches a particular size, then obviously after castration if we are to estimate the estrogen requirements of the animal, it is absolutely necessary to ascertain the minimum amount of estrogen necessary to cause the perineum to assume the same dimensions it attained normally before castration. We have seen from table 2, how this perineal response is affected by increasing amounts of estrogen. Even after assessing the amount of estrogen necessary to cause the perineum to reach its optimum size, it does not follow that this is the true estrogen optimum, it can only be regarded as the minimal optimum since any increase in the amount of estrogen will not be reflected to any extent commensurate with the speed with which the perineum reaches its maximum size nor with the actual size of the perineum. Once the minimum amount of estrogen necessary to produce optimum perineal turgescence is established, increasing this amount an hundred-fold does not cause any considerable difference in the size of the perineum (unpublished data). It is still possible that the estrogen requirements of the baboon are even greater than suggested by the experiments mentioned above.

The important fact established in this study is that the minimum optimum requirements for estrogen in the baboon are in general greater than those necessary to cause withdrawal bleeding. Moreover, it is in the optimum requirements rather than in the bleeding threshold that we see the greatest individual variation. In animals

without this sexual skin it is extremely difficult to determine the optimum level of estrogen effectiveness. At best our assessment of this level must be a crude approximation, because histological examination of the uterus and vaginal smears do not in any way provide any indication of optimum estrogen requirements. In rabbits, guinea pigs and rats this is a matter of the utmost importance as these animals are so frequently used to study the interaction of estrogen and progestogen on a quantitative basis and results so obtained, although useful, may not give precise information of how these two hormones interact in the normal animal. Nowhere in the literature is there information on the actual estrogen requirements of the rabbit, guinea pig, rat or mouse. In the latter two animals we certainly have many reports of the minimum amount of estrogen necessary to cause a positive vaginal smear but that is all.

In the case of the baboon we have already seen that in the majority of the animals there is a considerable difference between the estrogen values for bleeding and optimal perineal turgescence. It is highly probable that there may be a considerable difference also in rats, mice and guinea pigs, between the minimum amount of estrogen required to produce a positive vaginal smear and optimum estrogen effectiveness on the uterus. Either a new indicator may have to be elaborated for this purpose or else a much more critical histological and histochemical examination of the uterus and vagina may provide the solution to this problem. It is only after we have established beyond doubt the amount of estrogen required to produce a response as near as possible identical with that which the animal achieves by means of its own ovaries, that we shall be able to appraise quantitatively the hormonal requirements for a normal menstrual or estrous cycle in the lower animals, and in primates.

SUMMARY AND CONCLUSIONS

The estrogen requirements for production of withdrawal bleeding and minimal and optimal perineal responses, have been examined in 10 adult female baboons whose cycles have been accurately established before castration.

The minimal daily requirements for estradiol benzoate given over a period of 15 days to produce withdrawal bleeding was 0.01 mg., 0.02 mg. (3 animals), 0.03 mg. (1 animal), and 0.06 mg. (1 animal).

The perineum is sensitive to estrogen since in 3 animals a very slight perineal response was elicited in 3 animals after daily treatment with 0.005 mg. of estradiol. In 9 animals there was a slight response following daily injection of 0.01 mg. and the 10th responded to 0.02 mg. of estradiol.

The minimum daily amount of estrogen necessary to cause a perineal response similar to that produced by the baboon's own ovaries before castration was found to be 0.01 mg. for 1 animal, 0.02 mg. for 2, 0.03 mg. for 2 and 0.04 mg. for 5 animals.

Although there are slight individual differences in the amount of estrogen necessary to produce bleeding, this individual difference is accentuated with respect to the amount of estrogen necessary to cause optimal perineal turgescence.

It has been shown that there can be a marked discrepancy in the amounts of estrogen necessary to cause withdrawal bleeding and optimal perineal turgescence.

Evidence is adduced to show that the amount of estrogen necessary to cause optimum perineal turgescence in the baboon is a much closer approximation to the amount of estrogen elaborated normally by the baboon in the first half of the cycle than the bleeding threshold values.

Furthermore, it has been emphasized that in animals which do not possess the sexual skin, not only have the optimum requirements not been established, but also no techniques are available for this purpose.

The conclusion is drawn to the effect that the interaction of estrogen and progesterone on the normal menstrual or estrous cycle will be understood only when much more accurate data of a quantitative nature have been accumulated.

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ACTION OF DIETHYLSTILBESTROL IN THE CHICK[1]

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THE ACTION of diethylstilbestrol and estrone with respect to relative potency, and physiological activity has been considered at length in the mammal, and the literature is well reviewed by Lee et al. (1). Comparable studies, however, on other forms have been meager. It was demonstrated in our laboratory that estrogen has a very pronounced effect on the growth of the viscera in the White Leghorn cockerel and also greatly inhibits the secretion of androgen by the testes and gonadotropin by the pituitary (2). This experimental background has made it possible to analyze some aspects of the physiological action of diethylstilbestrol and to compare its effects with those of estrone. It was considered important to ascertain, in view of its estrogenic effect in mammals, if diethylstilbestrol would also duplicate the action of estrogenic hormone in the chick, namely: stimulate the growth of the viscera and inhibit testicular growth and androgen secretion.

MATERIAL AND METHODS

Several types of experiments were planned to check as fully as possible the physiological effects of diethylstilbestrol in the fowl. *a*) Chicks on a normal diet were compared with those on a limited diet. The latter were given food only on alternate days and in certain experiments to be noted in table 5, the food was available only part of the time on the feeding days. It has been shown (3) that such limited-diet birds secrete little, if any, pituitary gonadotropin. *b*) Pullets were also studied in order to eliminate as far as possible the action of endogenous androgen, and even more critical experiments of the same type were made with baby capons. These birds were caponized 4 days after hatching, using the technique previously described (4). *c*) Testosterone propionate was administered both alone and in combination with estrogen or diethylstilbestrol to determine the effect of the preparations on the response of the comb to exogenous androgen. *d*) Comparison of the comb weights of untreated and treated chicks also made it possible to measure the degree of inhibition of comb growth produced directly by the estrogens when given alone.

The combs were weighed when the chicks were autopsied and were removed by cutting at the base with fine pointed scissors. As in most of our previous work, organ weights are expressed in the tables as a percentage of body weight. All animals were without food for 12 hours previous to killing and for this reason the eviscerated

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[2] The diethylstilbestrol and estrogen used in this study were supplied through the kindness of Dr. J. P. Schooley, Director of Endocrine Research, Difco Laboratories, Inc., Detroit, Mich., and a grant-in-aid for research was given by this company.
Testosterone propionate was supplied by the courtesy of Dr. Erwin Schwenk, Director, Medical Research, Schering Corp., Bloomfield, N. J.
Technical assistance was given by the N. Y. A.

weight of the animals was not used in the tables. The method of calculating organ weights on the basis of organ weight/eviscerated body weight × 100 proved to be no more reliable than the usual calculation.

Both the estrogen and diethylstilbestrol were in sesame oil; the volume of the injections was 0.1 cc. when the preparations were administered subcutaneously and 0.02 cc. when administered in the comb. The testosterone propionate was also given in the same volume of sesame oil.

RESULTS AND DISCUSSION

The injection of diethylstilbestrol into cockerels had marked effect on comb and gonad weights and the adrenals were also affected. This action was similar to that previously noted for estrogen, but in order to make an adequate comparison, estrogen and diethylstilbestrol were injected concurrently into cockerels previous to the 30th day. These data are shown in table 1.

TABLE 1. 30-DAY-OLD COCKERELS INJECTED WITH ESTROGEN AND DIETHYLSTILBESTROL, 3RD TO 29TH DAY

Treatment[1]	No.	Body Weight gm.	Comb	Gonad	Adrenal	Thyroid	Pancreas	Liver	Intestine	
									Length	Weight
Estrogen										
2.5 I.U.	12	217.3	.3281	.0294	.0166	.00507	.429	2.62	47.4	4.41
5.0 I.U.	16	245.4	.3859	.0307	.0139	.00477	.433	2.65	44.2	4.36
100 I.U.	10	214.2	.2894	.0239	.0139	.00668	.504	2.49	46.4	4.26
500 I.U.	9	221.7	.1135	.0221	.0166	.00447	.483	2.93	46.9	5.09
Diethylstilbestrol										
0.0005 mg.	12	236.3	.3383	.0230	.0134	.00411	.385	2.39	43.0	3.76
0.005 mg.	12	226.0	.2740	.0243	.0145	.00435	.429	2.53	45.7	3.84
0.200 mg.	12	235.6	.0525	.0132	.0172	.00534	.438	2.98	44.0	4.16
Controls										
Untreated	30	272.3	.4910	.0316	.0127	.00513	.413	3.21	35.4	4.11

[1] Amount given daily.

Organ weights expressed as percentages of body weight, except for the length of the intestine which is expressed as centimeters per 100 m. of body weight.

The 2.5 and 5.0 I.U. dosages of estrogen inhibited comb growth but had very little influence on testes weights. The fact that the effect on the comb at the lower dose is more marked than at the higher, suggests that these dosages are approximately the threshold level for the hormone action. This conclusion is further supported by the observation that the weight of adrenal, pancreas, liver, are all essentially the same as the control averages. The administration of 100 and 500 I.U. of estrogen, on the other hand, did cause a change in the percentages for most of the latter organs. The comb and testes weights are greatly inhibited, particularly at the 500-unit dosage in which the comb is only 23.1 per cent and the testes 69.9 per cent of the control average. It is significant that the mean for the comb is almost identical with that of the capons shown in table 2. The heaviest estrogen dosage also produced an increment in the weights of adrenals, pancreas, and intestine and intestine length. This point is important when the diethylstilbestrol-treated birds are considered. The thyroid weights are discouragingly variable and no conclusion can be drawn from these

data concerning the effect of the female hormone on this organ. A future study of the cytology of the gland may help in this respect. It should also be noted that in all of the estrogen-treated series the body weight is decidedly lower than the control average.

The injection of diethylstilbestrol was made in terms of mg. of the crystalline preparation. It is apparent that diethylstilbestrol was much more potent in the inhibition of comb and gonad weights. The .0005 mg. dosage decreased the former to 68.9 per cent and the latter to 72.8 per cent of the control weights. The .200-mg. dose inhibited the growth of these two organs until the comb is only 10.7 per cent and the testes 41.8 per cent of the averages observed in the untreated chicks. Attention should be called again to the average for the capon comb in table 2, because the comb weight in the series which received the largest amount of diethylstilbestrol is only about 50 per cent of that in the capons. It was observed that the adrenal weights were increased in the estrogen-treated series and the weights in the diethylstilbestrol-treated group are even greater. The adrenal average in the .200 mg. series exceeds the control by 35.4 per cent. In contrast to these data, the response of the other organs is not significantly different in most instances. Indeed, instead of stimulation there is actually a decrease in the relative weights in several series.

It has been suggested that diethylstilbestrol is 32 times more potent in its estrogenic effect in the rat than is estrone (1), and it is interesting to compare our data in this respect. If 1 I.U. of estrogen is the equivalent of .0001 mg. of crystalline estrone, it may be noted that the .005 mg. of diethylstilbestrol produced comb, gonad, and adrenal responses almost exactly comparable to those observed in the 100 I.U.-estrone series. The diethylstilbestrol, therefore, would be about twice as potent as the estrogen. This comparison with the mammalian data is hardly justified, however, since we are considering an inhibition of development, whereas previous determinations were concerned with a positive response, namely: the stimulation of the rat or mouse vagina or uterus.

The failure of the various other structures to respond to diethylstilbestrol treatment suggests, however, that there is a fundamental physiological difference in the action of estrone and diethylstilbestrol. It was reported (2) that 1000 I.U. of estrone daily produced an increase of nearly 100 per cent in the alimentary canal of 30-day-old chicks. The .200 mg. dosage of diethylstilbestrol should be physiologically in excess of this dose; but in the series so treated, intestine length is the only measurement which exceeds the control values. The diethylstilbestrol, therefore, is not comparable to the estrogen in its action on the viscera, and .200 mg. actually has less effect on the viscera than the 500 I.U. of estrogen.

The question of toxicity has been a major item of consideration when diethylstilbestrol is given clinically and in experimental animals. There is little or no deleterious effect resulting from the injection of this preparation into the chicks at the dosage level used here. Body weights, which are a reasonably good index of the condition of the birds, were less than the control average but were actually greater than the mean weights of the estrone-treated animals. The diethylstilbestrol had no toxic effect in the chick.

Payne (5) has shown that basophilic cells were almost entirely absent from the pituitaries of the chicks which received estrone and diethylstilbestrol and the pituitaries were accordingly very different from those of the normal animals. This would account for the elimination of gonad-stimulating principle from the pituitary which in turn would account for the decreased androgen secretion by the testes. The difference in the response of the chick to these two estrogens denotes some interesting

physiological problems. It has been reported (6) that estrone acts directly on the comb to suppress its response to male hormone, and in view of the great inhibition produced by diethylstilbestrol, it would appear that this preparation has a similar effect. To test this possibility, chick-capons were injected daily with .005 mg. of diethylstilbestrol, with .04 mg. of testesterone propionate and with a combination of these two substances. Similar injections were made into normal-diet males at the same time to serve as check animals. The data are summarized in table 2.

TABLE 2. 30-DAY-OLD CAPONS AND COCKERELS GIVEN DIETHYLSTILBESTROL AND TESTOSTERONE PROPIONATE FROM THE 10TH TO 24TH DAY

Treatment[1]	No.	Body Weight, gm.	Comb	Gonad	Adrenal
Capons					
Untreated	11	200	.1187		.0177
.04 mg. Testosterone propionate	12	217.7	.9510		.0141
.005 mg. Diethylstilbestrol	12	192.0	.1280		.0159
.005 mg. Diethylstilbestrol+.04 mg. Testosterone propionate	11	194.9	.8989		.0163
Cockerels					
.04 mg. Testosterone propionate	14	205.3	.9954	.0204	.0151
.005 mg. Diethylstilbestrol	10	230.8	.3544	.0300	.0155
.005 mg. Diethylstilbestrol+.04 mg. Testosterone propionate	10	208.9	1.082	.0225	.0168

[1] Amount given daily.
Organ weights expressed as percentages of body weight.

Testosterone propionate produced a net increment in the capon comb of 709 per cent and, surprisingly, the diethylstilbestrol also caused a slight increase in comb size. Because of these observations, the combination injections are especially significant. When the two preparations were administered simultaneously, the comb size exceeded that of the untreated capons by 657 per cent. Although this is somewhat less than the response to testosterone propionate it is not significantly so in view of the number of animals used, and also by comparison with the results in the cockerel series.

Male hormone produced a slightly larger comb in the cockerels than it did in the capons, but it should also be noted that there was a very marked suppression of the gonads when compared with the control data shown in table 1. It is tempting to speculate that this amount of male hormone, acting through the pituitary, may have suppressed almost entirely the endogenous androgen. This would account for the similarity of the data with the results in the capon series. Diethylstilbestrol inhibited the comb growth, but the testis weights were only slightly modified. The injection of both androgen and diethylstilbestrol produced combs which were slightly larger than those observed when the androgen was given alone. There was also an inhibition of the testes but this was not as great as in the testosterone propionate-treated series. Whether this variation is significant cannot be answered at this time.

These data leave little doubt that the effect of diethylstilbestrol on the comb is secondarily acting through the pituitary to inhibit testicular secretion of male homone. Both the capons and cockerels show that the exogenous androgen is about equally effective either in the presence or absence of diethylstilbestrol. It was note-

worthy, in table 1, that the highest dosage of diethylstilbestrol, .200 mg. daily, decreased the comb size in the normal bird to less than that of the capon. This suggests that there must be an extra-gonadal source of a comb growth-promoting substance in the chick and that this substance is eliminated by the high dosage of diethylstilbestrol. Witschi has demonstrated (7) that pituitary hormones have an effect on secondary sex characters of the Weaver Finches, but evidence is lacking that anterior pituitary hormones will stimulate comb growth in the chick. It is possible, however, that other glands may exert an influence on the comb. Androgen activity has frequently been ascribed to the adrenal cortex and the adrenal gland undoubtedly is affected by diethylstilbestrol treatment. It is also possible that the thyroid gland may be involved, because it has been shown in our laboratory (Hale, unpublished data) that crystalline thyroxin augments the response of the comb to androgen treatment. The problem is one which affords interesting possibilities for continued study.

Attention should be called to the variations noted in the adrenal weights of the capons (table 2) and the controls (table 1). The capon adrenal averages nearly 40 per cent heavier than that of the untreated controls. When the male hormone or diethylstilbestrol was administered to these birds, the adrenal weights were decreased. It had been thought that the increase in adrenal wieght observed in the experiments in table 1 and recently reported for mammals (8), might have been in the nature of an 'alarm' reaction and did not involve the pituitary gland to any considerable extent. The fact that the adrenal averages were decreased in the injected capons suggests that the action is one which may entail a very fundamental change in the pituitary secretion. The data are also interesting in light of the earlier suggestion that the cockerels, data for which are in table 2, were essentially 'hormone castrates,' because there is a very close correlation with the adrenal weight in the capon series. This is especially noteworthy for the weights in the diethylstilbestrol and combination series which differ from each other by only .004 per cent and .005 per cent, respectively. This may be a mere coincidence but is worthy of further study.

It is possible to make an additional analysis of the action of diethylstilbestrol on comb growth by using pullets and limited-diet chicks. The secretion of androgen or comb-growth promoting substance in such animals is very small in amount (4), and therefore the problem of endogenous hormone is minimized. Three experiments of this type were made and the data are presented in tables 3, 4, and 5.

TABLE 3. 15-DAY-OLD LIMITED-DIET CHICKS INJECTED WITH DIETHYLSTILBESTROL, ESTROGEN AND TESTOSTERONE PROPIONATE FROM THE 3RD TO 14TH DAY

Treatment[1]	No.	Body Weight, gm.	Comb
Untreated	23	63.2	.0365
.02 cc. Sesame oil, comb+0.1 cc. sesame oil, neck	25	63.0	.0895
20 I.U. Estrogen, comb+0.1 cc. sesame oil, neck	26	61.1	.0714
.01 mg. Testosterone propionate, neck+.02 cc. sesame oil, comb	25	63.1	.1659
20 I.U. Estrogen, comb+.01 mg. Testosterone propionate, neck	23	61.7	.1439
.005 mg. Diethylstilbestrol+.01 mg. testosterone propionate, neck+0.1 cc. sesame oil, comb	24	63.6	.1543

[1] Amount given daily.
Comb weight expressed as percentage of body weight.

Sesame oil was injected into the comb alone or as a carrier for the male hormone, and in a similar fashion oil was injected into the neck. It is important to note that the sesame oil produced an increment of the comb in the chicks which received no hormone. This is undoubtedly the result of the increased pressure, the presence of small amounts of oil which are not absorbed or removed completely by the blotting when the comb was excised, and probably to some irritation of the tissues by the injection. Administration of 20 I.U. of estrogen, however, definitely decreased the comb size, the mean is 17 per cent less than the sesame oil-injected controls. The injection of testosterone propionate into the neck and of sesame oil into the comb, increased the comb weight by 88.6 per cent. When the estrogen and male hormone were given simultaneously, however, the percentage increase over the controls was only 60.8 per cent. This figure is interesting because estrogen alone decreased the comb by 17 per cent, when administered with the androgen, the response was 27.8 per cent lower than the average for the androgen-treated birds. Diethylstilbestrol apparently also had some inhibitory effect in these experiments, but it was less than that produced by the estrogen; the comb average in the combination series is about 93 per cent of that in the male hormone-treated group. These experiments in general are comparable with those recorded in table 2, inasmuch as the diethylstilbestrol appears to have slight, if any, direct effect on the comb, but estrogen does appear to have direct inhibitory action.

A second type of experiment accordingly was undertaken. Normal-diet pullets were given a uniform amount of testosterone propionate in the comb on alternate days, and at the same time sesame oil was injected into the neck, either alone or with varying amounts of diethylstilbestrol. The daily dosages of diethylstilbestrol ranged from .0005 to .01 mg.

TABLE 4. 16-DAY-OLD NORMAL-DIET PULLETS RECEIVING TESTOSTERONE PROPIONATE AND DIETHYLSTILBESTROL

(Injected on days 6, 8, 10, 12, 14, also prime injection of .01 mg. of testosterone propionate in neck on 4th day)

Treatment[1]	No.	Body Weight, gm.	Comb	Gonad	Comb per Gonad Ratio of .0200%
.002 mg. Testosterone propionate, comb 0.1 cc. Sesame oil, neck	15	87.3	.1324	.0262	.1011
.002 mg. Testosterone propionate comb .0005 mg. Diethylstilbestrol, neck	15	89.9	.1132	.0218	.1039
.002 mg. Testosterone propionate, comb .001 mg. Diethylstilbestrol, neck	16	82.5	.1395	.0240	.1163
.002 mg. Testosterone propionate, comb .005 mg. Diethylstilbestrol, neck	15	85.3	.1159	.0203	.1142
.002 mg. Testosterone propionate, comb .010 mg. Diethylstilbestrol, neck	15	81.9	.1066	.0182	.1171

[1] Amount given daily.
Organ weights expressed as percentages of body weight.

The comb percentages are remarkably uniform, ranging from .1324 in the chicks receiving androgen-sesame oil to .1066 in those receiving androgen and .01 mg. of diethylstilbestrol. The maximum comb size was observed in the group which was given .001 mg. of diethylstilbestrol with the male hormone. Fortunately gonad

weights were taken and some additional analysis can be made of the data in these series.

It has been demonstrated (9) that the pullet comb grows more rapidly than does the capon comb, indicating the presence of some comb-growth principle possibly released by the ovary. It is reasonable to assume that the inhibitory effect of diethylstilbestrol on the pituitary would reduce ovarian size and inhibit to some extent the secretion of any comb-growth substance by that organ. The ovaries are smaller in the diethylstilbestrol-treated series and, with the exception of the .001-mg. series, become progressively lighter as the dosage is increased. It was thought that an adjustment for this size variation of the ovaries might correct or lessen the differences which were observed between the series. A method of making comb-weight corrections on the basis of a uniform weight of testes was employed in an earlier study (3), and accordingly the comb percentages were calculated for a constant ovarian/body percentage of .0200. These adjusted averages are shown in the last column of table 4, and there is certainly a most remarkable uniformity of the data. The range is from .1011 for the control to .1171 for the series receiving .01 mg. diethylstilbestrol and the results are even more significant when it is remembered that these are normal-diet birds in which variation is usually greater than in the limited-diet chicks.

TABLE 5. 15-DAY-OLD LIMITED-DIET PULLETS INJECTED WITH DIETHYLSTILBESTROL AND TESTOSTERONE PROPIONATE FROM THE 3RD TO 14TH DAY

Treatment[1]	No.	Body Weight, gm.	Comb	% Increase
.02 cc. Sesame oil, comb+0.1 cc. Sesame oil, neck	13	46.7	.0964	
.01 mg. Testosterone propionate, neck+.02 cc. sesame oil, comb	15	47.5	.1684	74.7
.005 mg. Diethylstilbestrol, comb+0.1 cc. sesame oil, neck	13	48.2	.1226	27.2
.005 mg. Diethylstilbestrol, comb+.01 mg. testosterone propionate, neck	15	47.1	.1917	98.8

[1] Amount given daily.
Comb weight expressed as percentage of body weight.

The question might be raised concerning the reliability of the ovarian weight correction for the data, and for this reason an additional group of experiments was made in which limited-diet pullets were used exclusively. These birds were placed on an extremely restricted food intake and were especially uniform in response, even though the number of chicks used was not as great as in some of the other groups.

The injection of sesame oil into the comb and testosterone propionate into the neck of these limited-diet pullets resulted in a comb increment of 74.7 per cent. The injection of diethylstilbestrol into the comb and the sesame oil into the neck produced an increase in the comb of 27.2 per cent. This is noteworthy because it was observed in table 3 that estrogen actually decreased comb size under similar conditions. Administration of the two preparations at the same time produced combs which averaged 98.9 per cent greater than those of the androgen-sesame oil-injected chicks. This response is, therefore, nearly equal to the expected size on the basis of the combined effects produced by the two preparations when administered separately. The expected increase for the combination injections should be 101.9 per cent; only 3.1 per cent greater than that actually produced by the injections.

These data on the limited-diet pullets seem ample support for the ovarian correc-

tion applied in table 4 and, furthermore, they demonstrate that diethylstilbestrol does not have a direct inhibitory effect on the comb. These results in conjunction with those already noted in tables 2 and 4, demonstrate that under certain experimental conditions diethylstilbestrol will actually stimulate comb growth.

SUMMARY

The administration of diethylstilbestrol and estrogen to normal-diet chicks inhibited body growth slightly, and diethylstilbestrol had a very marked inhibitory action on the development of comb and gonads. The adrenal glands were hypertrophied in the diethylstilbestrol-injected series, but the weight of liver, pancreas, and intestine and the intestine length was not significantly greater than in the control. In this respect, the action of the diethylstilbestrol is different from estrogen. Injection of diethylstilbestrol into capons, alone and in combination with testosterone propionate, showed that the effect on the comb was a result of suppressing the secretion of endogenous androgen. It was noted, however, that the administration of .200 mg. daily produced combs in normal chicks which were only 50 per cent of the size of the capon combs. The possibility of the presence of extra-gonadal comb-growth-promoting substance in the chick is considered.

Treatment of pullets and limited-diet chicks further support the hypothesis that the action of diethylstilbestrol on the comb is indirect. Pullets given dosages of diethylstilbestrol ranging from .0005 to .010 mg. in combination with androgen have combs which do not differ significantly. Pullets on extremely limited-diet show that diethylstilbestrol will actually stimulate some comb-growth when administered directly into the comb. It is also noteworthy that when diethylstilbestrol and testosterone propionate were given at the same time in these chicks, the comb response was almost exactly the same as the summation of the effects of these two preparations when given separately.

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EFFECT OF PROGESTERONE AND DESOXYCORTICOSTERONE ON ACCESSORY SEX ORGANS OF THE CASTRATE MALE GUINEA PIG

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SEVERAL INVESTIGATORS (1–3) have recently shown by direct evidence that progesterone, the corpus luteum hormone, has the capacity to induce androgenic reactions in the rat. Previously, evidence indicative of the androgenicity of progesterone had been suggested by several investigators. Hill (4–5) showed that ovaries of mice, when transplanted to the ears of castrate mice, apparently produced an androgenic substance. This work was confirmed by Deansely (6) in the rat. Papanicolaou (7, 8) also showed that ovaries in normal guinea pigs had an androgenic action when stimulated with the APL factor of pregnancy urine. Several other workers have also shown that ovaries, transplanted into a large series of castrate male rats or guinea pigs, had an androgenic effect in an occasional animal (9, 10, 11).

Since the only direct evidence for the androgenicity of progesterone had been obtained in the rat, it was thought of interest to determine directly if this property could also be shown with the guinea pig as the experimental animal.

It is also becoming more evident that, under certain circumstances, the adrenal glands exert an androgenic action. It is well known that various degrees of masculinization accompany adrenal cortical tumors in women and that clinical symptoms of virilism, as well as the excretion of androgens, regress following the removal of the adrenal tumors. In the immature rat and mouse (12, 13, 14) castration is not followed by complete atrophy of the prostate and seminal vesicles without adrenalectomy.

The means by which the androgenic effects of the adrenal are mediated are not as yet clear. Hence, it was thought of interest to determine whether or not the action of desoxycorticosterone acetate might also be similar to that of the sterols of the adrenal cortex which possess some androgenic action in the guinea pig. A preliminary report of the results was published in abstract form (15).

METHODS AND RESULTS

Forty immature male guinea pigs weighing between 192 and 200 gm. were selected for the following series of experiments. Thirty-two animals were castrated and the remaining 8 were killed as normal controls at the beginning of the study. From 10 days to 3.5 months following castration the various injection experiments were initiated. This time allowed for complete atrophy of the accessory reproductive organs (prostate, seminal vesicles) in a large percentage of animals.

In one group of animals, daily subcutaneous injections of 1 mg. of crystalline progesterone[1] in oil were administered for a 12-day period to each of 10 animals. In another series, daily subcutaneous injections of 3 mg. of desoxycorticosterone ace-

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[1] The progesterone was supplied by Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products Summit, N. J.

tate[2] were given for a 12-day period to 10 castrate guinea pigs. Six castrate animals, used as controls, were injected with 1 cc. of sesame oil daily for a similar period and 6 animals were also each injected with 5 mg. of testosterone propionate daily.

Twenty-four hours after the last injection all of the animals were killed. Body weights as well as the weights of the prostate glands and seminal vesicles were recorded. The prostates and seminal vesicles were fixed in Bouin's solution, embedded in paraffin, sectioned serially and stained with hematoxylin and eosin. All sections were cut at 7μ and the epithelial cell height of a representative number of cells in every 5th section was measured by means of an ocular micrometer.

The data on changes in body weight and weights of the prostate glands and seminal vesicles for each series of guinea pigs are shown in table 1. It can be noted that in all 4 of the injected series there was a consistent weight gain for the 13-day period. The differences in weight increase between the various series was not within the border of statistical significance and hence must be considered a normal variation. The series receiving desoxycorticosterone acetate showed the smallest increase in weight. This seemed reasonable on the basis that excessive amounts of desoxycorticosterone acetate actually produce a body weight decrease as shown by Ingle, Higgins and Kendall (16).

TABLE 1. EFFECT OF VARIOUS STEROIDS UPON ACCESSORY SEX ORGANS OF CASTRATE MALE GUINEA PIGS

Series	No. of Animals	Initial Body Wt.	Final Body Wt.	Total Inject. Per Animal	Prostate		Seminal Vesicles	
					Weight mg.	Epith. Height, μ	Weight mg.	Epith. Height, μ
Normal[1]	8	200	200		110.0	6.7	98.0	10.72
Sesame Oil	6	198	273	12 cc.	115.0	6.85	93.0	11.56
Progesterone	10	192	288	12 mg.	430.0	22.45	378.0	23.20
Desoxycorticosterone Acetate	10	197	261	36 mg.	148.0	9.98	122.0	13.13
Testosterone	6	200	342	60 mg.	730.0	23.88	560.0	27.76

[1] Animals not castrated.

With regard to the weight of the prostate glands in the injection series, it can be seen (table 1) that the progesterone and the testosterone-injected series were the only two series which showed a weight gain of statistical significance, when compared with the normal control series. While these weight increases were indicative of a stimulating action not only of testosterone but also of progesterone, the indications were more clearly in evidence upon histological examination. Figure 1 shows a section through the prostate of an immature and inactive prostate. In this gland the nuclei were darkly stained and the cells were flattened or cuboidal. Figure 2 shows the condition of the prostate from a desoxycorticosterone acetate-injected animal. Here the prostate again appeared histologically similar to the non-active gland. However, figure 3, which is a photograph of a section through the prostate of a progesterone-injected animal, showed evidence of some stimulatory activity. The cells were more columnar and the nuclei more lightly stained. Measurements of the secretory epithelium of the prostate of all series (table 1) showed that the greatest increase was found in the testosterone-injected series. This was to be expected. However, the height of the epithelium in the progesterone series also showed increases which were

[2] The desoxycorticosterone acetate and testosterone propionate used in these experiments were supplied by the Schering Corp., Bloomfield, N. J.

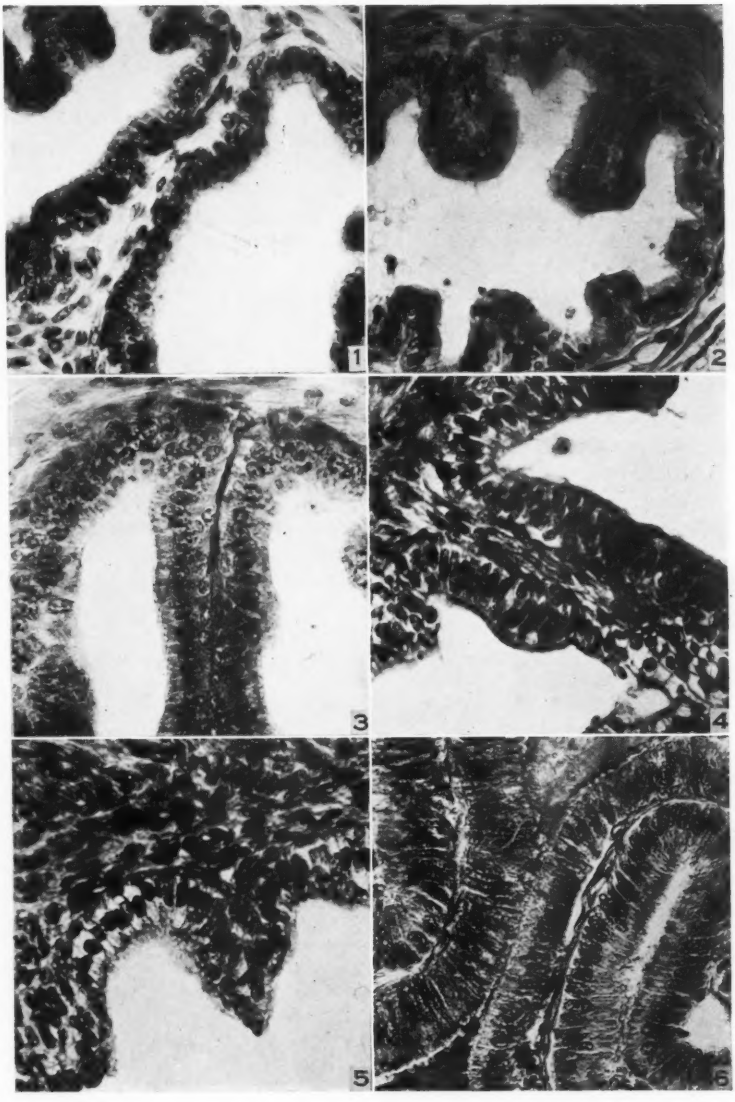

Fig. 1. PROSTATE OF AN IMMATURE MALE GUINEA PIG weighing 200 gm. ×240.
Fig. 2. PROSTATE OF A CASTRATE GUINEA PIG after treatment with desoxycorticosterone acetate.
Fig. 3. PROSTATE FROM A CASTRATE GUINEA PIG after treatment with crystalline progesterone. Note the difference in cell height and secretory granules of the epithelium when compared to figure 1 and 2.
Fig. 4. SEMINAL VESICLE of a normal immature guinea pig. ×240.
Fig. 5. SEMINAL VESICLE OF CASTRATE GUINEA PIG treated with dexoxycorticosterone acetate. ×240.
Fig. 6. SEMINAL VESICLE OF CASTRATE GUINEA PIG after treatment with progesterone. ×240.

of statistical significance. In general, the histological studies showed that the prostate glands of the animals receiving progesterone and testosterone had tubules which were distended with secretion and that the secretory epithelial cells had increased in height with the nuclei increasing in size and taking a lighter stain. As compared to the testosterone-injected series, the prostates of animals receiving desoxycorticosterone acetate showed tubular alveoli which were smaller and shorter, the tubular diameter being much reduced and the epithelial cells more cuboidal.

The weights of the seminal vesicles in the series receiving progesterone and the one receiving testosterone showed a definite increase which could not be considered within the range of normal variation when compared to the control series. While the weights of the seminal vesicles of the desoxycorticosterone acetate-injected series gave evidence of a slight increase above that of the control series they were not within the range of statistical significance. The histological sections of the seminal vesicles in the series receiving desoxycorticosterone acetate (fig. 5) also showed very little evidence of stimulation when compared to the control series (fig. 4). In this series the seminal vesicles all seemed to be characteristically inactive. Here the cavity of the gland was small, with little or no stainable material and the blind outpouchings were not extensive. Furthermore, the epithelial cells were low cuboidal and very few secretion granules seemed to be present.

As compared to the histologically inactive seminal veiscles described above, the seminal vesicles of the progesterone-injected series (fig. 6) were considered to be active. Here the cavity of the gland was enlarged, the villi were much more developed and a characteristic single layer of tall epithelial cells was present. The epithelium was uniformly granular with the nuclei showing coarse granules and the cytoplasm was made up of fine granules.

DISCUSSION

The results described above show that in the castrate male guinea pig, the injection of crystalline progesterone will regenerate involuting male accessory glands as well as maintain active secretory activity. Hence, this compound must be considered androgenic in action in the guinea pig as well as in the rat as has already been shown (1, 2, 3). That the quantity of progesterone need not necessarily be enormous to evoke the androgenic response, has been shown by the fact that only 1 mg. per day was injected for 12 successive days. This dosage produced a weight increase in the prostate of over 3 times the control weight as well as an increase of epithelial cell height which was nearly 4 times that in the normal control.

While it seems to be a well-established fact, at least in the rat, that the prostate gland responds more readily to lower doses of androgen than do the seminal vesicles, this fact was not verified in the present experiments. Here the comparative response of the seminal vesicles to the progesterone seemed to be as good as the response of the prostate gland to androgenic factors. However, it is possible that the seminal vesicles of the guinea pig have a lower threshhold of response to androgen than similar structures in the rat.

Many biological activities have been attributed to crystalline desoxycorticosterone since its isolation from the adrenal gland. This substance has been subjected to the accepted biologic tests for androgenic activity along with other activity tests and conflicting results have been reported. Hooker and Collins (17), using synthetic desoxycorticosterone acetate, have presented experimental evidence to show that desoxycorticosterone was capable of exerting androgenic effects. However, these investigators have based their evidence on the increase in seminal vesicle weight in

two rats treated with desoxycorticosterone acetate over that of one control rat. In addition, they reported on 6 capons treated at various dosage levels. In this case their results showed that desoxycorticosterone acetate was only 1/33 as active as androsterone in stimulating comb growth. These experiments have since been repeated (18, 19) and no androgenic activity for desoxycorticosterone acetate was found either in the castrate rat or in the chick. The results, as presented in this paper, of testing desoxycorticosterone acetate for its androgenicity in the guinea pig also failed to show any of the effects which are ordinarily attributed to male sex hormones.

On the basis of the experiments presented here progesterone may be considered androgenic in action. As to whether progesterone acts directly on the male accessory organs or whether it is converted to an androgen by the body directly or indirectly through an intermediate structure, still remains a question. With regard to the chemically related compound, desoxycorticosterone acetate, the experiments fail to show, in the doses administered, that this compound is androgenic in the male guinea pig.

SUMMARY

The purpose of this study was to determine the effect of progesterone and desoxycorticosterone acetate on the accessory sex organs of immature castrate male guinea pigs.

Daily subcutaneous injections of 1 mg. of crystalline progesterone in oil were administered for a 12-day period to one series of castrate guinea pigs. To another series, daily subcutaneous injections of 3 mg. of desoxycorticosterone acetate were given for a 12-day period. Some controls were injected with sesame oil for a similar period, while others were injected with testosterone propionate. A few animals were killed as normal controls. Twenty-four hours after the last injection, the body weights of all animals were recorded and the animals killed. Weight records, histological studies and epithelial cell measurements of the prostates and seminal vesicles were made. The following conclusions have been reached. *a*). Crystalline progesterone was capable of regenerating involuting male accessory sex organs as well as maintaining secretion in these in the castrate male guinea pig. Hence, progesterone was considered to be androgenic. *b*). Desoxycorticosterone acetate, in the dosages administered, failed to elicit an androgenic response in the accessory sex organs of the male guinea pig and was not considered to have an androgenic action.

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ANTI-FIBROMATOGENIC ACTION OF A NATURAL CORTICAL HORMONE (DEHYDROCORTICOSTERONE OF KENDALL)[1]

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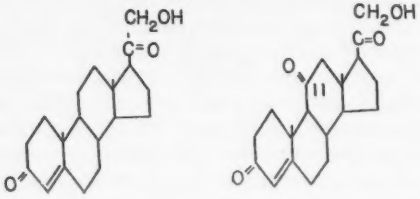
LIPSCHÜTZ and Iglesias (1, 2) have reported that subserous fibroids of the uterus and of different abdominal organs, or of the abdominal wall, can be elicited in the female guinea pig by estrogens. Uterine fibroids have been reported first by Nelson (3, 4), Moricard and Cauchoix (5). The findings of Lipschütz and Iglesias referring to extragenital fibroids have been corroborated and amplified by the work of Lipschütz, Vargas, Koref and others (6) in this Department, and likewise by Sammartino and Gandolfo (7), L. M. Woodruff (8) and Von Wattenwyl (9).

As has been shown (10, 11), these experimental fibroids can be prevented by different steroids such as testosterone, progesterone and desoxycorticosterone. The



Desoxycorticosterone Dehydrocorticosterone

Fig. 1

statement that a cortical hormone can be active as an antagonist against the fibromatogenic acton of estrogens is of considerable interest. It has also been demonstrated (12, 13) that the adrenal cortex, and also desoxycorticosterone, decrease the vaginal sensitivity of castrated rats to continuous stimulation with estrogens. One may tentatively assume that the adrenal cortex interferes in the sexual rhythm and that disturbances of the metabolism of cortical steroids play some part in the pathology of the genital tract including atypical proliferation. But it must be emphasized that desoxycorticosterone differs from the remaining cortical steroids in the lack of an oxygen atom in position 11 (14). The question arises whether cortical hormones with an oxygen in postion 11 (fig 1) will also be able to exert an anti-fibromatogenic action. This question is

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of fundamental interest since all cortical steroids to which some part of the endocrine function of the adrenal cortex may be ascribed (15), possess two structural peculiarities, which are also relevant for the anti-fibromatogenic action of steroids (16): *a*) the ketonic oxygen in position 3 which is essential for anti-fibromatogenic action, and *b*), the short side chain of two carbons in position 17 by which the anti-fibromatogenic activity of steroids is, in the guinea pig, greatly enhanced when judged from the point of view of the 'anti-fibromatogenic threshold,' or the daily minimum quantity necessary to prevent fibroids (17).

EXPERIMENTS AND RESULTS

Cylindrical pellets of crystalline dehydrocorticosterone (18) which weighed 4.5 to 8.1 mg. were implanted under the skin of castrated female guinea pigs simultaneously

Fig. 2. Uteri of 6 animals of the *estradiol group* (*1* to *7*). Duration of experiments: 70 to 73 days. Weight of animals when implanted with pellets: 340 to 450 gm. Premature complete absorption of estradiol in *1* but tumors present (not photographed; total tumor effect, 3.5). Mesometrial tumors especially in 3 (*class 2*), *4* (*class 3*) and *7* (*class 3*, not all visible in the photograph). Apical tumors in all animals but especially in *4* (*class 3*) in which a tumor of the abdominal wall above the right apical tumor also is to be seen (black arrow, *class 2*). Fig. 3. Uteri of 7 animals of the *desoxycorticosterone acetate-estradiol group* (*10* to *16*). Weight of animals when implanted with pellets: 350 to 520 gm. Duration of experiments: 70 to 73 days. No uterine or apical tumors.

with pellets of estradiol (2.9 to 5.6 mg.). A group of animals implanted with estradiol alone (2.1 to 5.0 mg.), and a third group with estradiol (2.9 to 5.1 mg.) and desoxycorticosterone acetate (4.5 to 9.1 mg. of the ester, or 3.9 to 8.1 mg. of the free hormone) were used for comparison. Necropsy was made 60 to 73 days after implantation. The pellets were recovered, dried at room temperature in vacuum and weighed. The results are given in table 1.[2]

[2] Part of these experiments has been communicated in the Sociedad de Biología de Santiago.

The genital bleeding elicited by estrogens was prevented by dehydrocorticosterone as it was by desoxycorticosterone acetate. Closure of the vagina was more frequent with desoxycorticosterone acetate than with dehydrocorticosterone. Increase in the size of the uterus was less when the cortical steroids were added to estradiol than with estradiol alone (fig. 2 to 4).[3] Fibroids, both uterine and extragenital, were prevented by desoxycorticosterone acetate in the usual way; only occasionally there were, in the group treated with desoxycorticosterone acetate, uterine and abdominal tumors of about 1 mm. in diameter, i.e. of *class 1* or more, according to our system of classification (19, 20); for examples, see also figures 2 and 4. On the contrary, these tumors were present in 7 of 10 animals of the group treated with dehydrocorticosterone, but they were small, in general, and the total tumoral effect in this group was

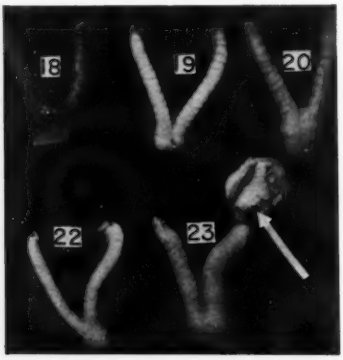

Fig. 4. UTERI of 5 animals of the *dehydrocorticosterone-estradiol group* (18 to 23). Weight of animals when implanted with pellets: 350 to 480 gm. Duration of experiments: 69 to 71 days. Small apical tumors (*class 1*) in 19 to 22; big apical tumor of *class 3* in 23. Also minute subserous nodules related with hypertrophied ventral ridge of uterus and minute mesometrial tumors (*class 0.5*) in 23.

considerably inferior to the average for the estradiol group. Only one animal of the group receiving dehydrocorticosterone reached the average for the total tumoral effect of the estradiol group as against 6 in the latter (see table 1).

The hypertrophy of the mammary glands was as great in the groups receiving estradiol and dehydrocorticosterone or desoxycorticosterone acetate as in the estradiol group, or even greater.

Neither desoxycorticosterone acetate nor dehydrocorticosterone produced masculinization of the clitoris. In our experiments with these substances there was no hypertrophy of the corpora cavernosa and no development of the horny styles, i.e. no hypospadic penis-like organ as described in the guinea pig with testicular graft (21, 22) or described as occurring as an intersexual aberration in otherwise normal female guinea pigs (23). On the contrary, the penis-like organ was elicited with testosterone (10, 24) and dehydrotestosterone (unpublished). It is evident that the antifibromatogenic action is not necessarily concomitant with the masculinizing one (16).

DISCUSSION

The experiments show that dehydrocorticosterone is, like desoxycorticosterone,

[3] A description of the comparative microscopical condition of the uterine wall shall be given elsewhere, together with data referring to other anti-fibromatogenic steroids.

anti-fibromatogenic, i.e., the ketonic group in position 11 is not contrary to this activity. We are as yet unable to say whether the anti-fibromatogenic activity is diminished by oxidation in position 11 as it would seem according to table 1. The 'anti-fibromatogenic threshold' or the minimum quantity absorbed per day, above which fibroids of *class 1* were absent (17), was certainly greater in the group receiving dehydrocorticosterone than in the group receiving desoxycorticosterone acetate; fibroids were present in animals receiving 110 to 114 μg. of dehydrocorticosterone per day, whereas they were always absent with more than 72 μg. of desoxycorticosterone acetate per day. But it must be insisted that the difference may be due to the fact that in 9 of 10 cases the absorption of the dehydrocorticosterone pellet was completed

TABLE 1. EXPERIMENTS IN 31 CASTRATED FEMALE GUINEA PIGS WITH SUBCUTANEOUSLY IMPLANTED PELLETS OF ESTRADIOL, DESOXYCORTICOSTERONE ACETATE AND DEHYDROCORTICOSTERONE. Duration of experiments 60–73 days, group average 66 to 73 days

Series XXXII	Absorbed		Total Tumoral[4] Effect		Number of Animals				Absorption of Cortical Steroid per Day,
Group	Estradiol, mg.	Cortical steroid, mg.	Individual animals	Average	Total	With individual tumors, *class 1* or more	Reaching average total tumoral effect of estradiol group 5.0	With genital bleeding	μg.
Estradiol	1.8 (0.8–3.0)[1]	0	1, 1.5, 1.5, 3.5, 4, 5, 6, 7, 8, 9, 12	5.3	11	9	6	3	
Estradiol and desoxycorticosterone acetate	1.5 (0.6–3.1)	4.8[2] (3.4–7.9)	1, 1, 1, 1, 1.5, 1.5, 1.5, 2, 2, 6.5	1.9	10	3	1	0	With tumors, *class 1* 59–72 Without 50–100
Estradiol and dehydrocorticosterone	1.9 (1.0–3.3)	6.4[3] (4.6–8.1)	0.5, 1, 1, 1[0], 1.5[0], 2.5, 3.5, 3.5, 4.5, 5	2.4	10	7	1	0	With tumors, *class 1* 74–114 Without 67–100

[1] Figures in parenthesis indicate range.
[2] Figures given for *free* desoxycorticosterone; complete absorption in 3 cases.
[3] Complete absorption in 9 cases.
[4] The 'total tumoral effect' represents the sum of 4 numerical values (0.5 to 3) relating to the size and number of the tumors a), of the uterus (subserous, mesometrial), b) of the mesosalpinx or 'apical' tumors, c) of the digestive tract and abdominal wall, and d) of the spleen (19, 20). For graphic examples of tumors of *classes 1* to 3 see figure 2 and 4 of the present paper; for examples of class 0.5 see references 20, 25.—Total tumoral effect of 0.5 to 1.5 was due to fibrous strands or minute nodules in different regions (10) and not to individual tumors of *class 1*, with the exception of those cases of the dehydrocorticosterone group which in this column are indicated with [0] where the value 1 to 1.5 corresponds to individual apical tumors.

before necropsy was made, whereas this occurred in only 3 of 11 animals with desoxycorticosterone acetate pellets. For this reason the anti-fibromatogenic threshold could not be calculated in an exact way for the group receiving the dehydrocorticosterone. It must also be pointed out that desoxycorticosterone has been used as the acetic ester, whereas dehydrocorticosterone was employed as the free hormone.

SUMMARY

Dehydrocorticosterone, a natural, chemically pure cortical hormone, was able to prevent the occurrence of uterine and other abdominal fibroids elicited by estrogens in the female guinea pig.

The anti-fibromatogenic activity was concomitant with certain anti-estrogenic actions, such as inhibition of uterine overgrowth and of genital bleeding, whereas the mammary gland was developed no less than with estradiol alone.

Dehydrocorticosterone behaved in its anti-fibromatogenic effects like synthetic desoxycorticosterone, progesterone and testosterone.

No exact quantitative statements are as yet possible about the anti-fibromatogenic threshold of dehydrocorticosterone.

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EFFECTS OF LACTOGEN ON NORMAL AND ADRENALECTOMIZED FEMALE RATS

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THE LACTOGENIC HORMONE, or a closely related fraction in the pituitary-lactogenic preparations, has been reported to cause maintenance of the corpus luteum (which apparently secretes progestin) in the rat as shown by *a*) suppression of the estrous cycle (1–4); *b*), implantation and maintenance of pregnancy after hypophysectomy (5, 6); and *c*), the production of traumatic placentomata (7–8). Since it has been reported (9–21) that injections of progestin, or the secretion of the corpora lutea of pseudopregnancy, pregnancy and lactation will prolong the survival time of adrenalectomized animals, the following experiment was tried to determine whether the secretion of lactogen-maintained corpora lutea would have a similar life-sustaining effect in adrenalectomized rats.

Treated-normal animals. To determine the potency of the lactogens used, 25 normal female rats (average initial body weight 150.2 gm.) were given daily subcutaneous injections of prolactin[1] in the dosage and for the periods shown in table 1, and were killed 1 to 2 days after the last injection. Body weights and vaginal smears were recorded daily, beginning 2 weeks prior to treatment and continuing throughout the experimental period. At autopsy the adrenals and ovaries were weighed. These organs, together with samples of the uteri, were fixed in Bouin's or Lavdowsky's solution for histological study.

TABLE 1. NORMAL ANIMALS RECEIVING DAILY SUBCUTANEOUS PROLACTIN INJECTIONS

Group	Number of Animals	Dosage			Average Weight At Autopsy		
		Total I.U.	Single injection, I.U.	Days	Total body, gm.	Ovaries, mg.	Adrenals, mg.
1[1]	6	3	60	10	6	137.0	45	
		3	80	20	4	153.3	58	51
2[2]	19	4	133	16.6	8	162.2	48	47
		5	140	20	7	179.8	51	47
		5	200	25	8	167.8	52	48
		5	250	25	10	177.6	52	49
3	15	controls				161.8	46	42

[1] Group 1 Difco prolactin, assayed to contain 10 I.U./mg.
[2] 2 Schering prolactin, assayed to contain 100 I.U./cc.

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[1] The prolactin was generously supplied by Difco Laboratories, Inc., Detroit, Mich., and The Schering Corp., Bloomfield, N. J.

Except for 3 rats which came into estrus during the period of treatment, the injected animals completed their cycles to, and remained in, diestrus (if not in that phase of the cycle at the time of the first injection.)[2] Their ovaries were 10.1 per cent[3] heavier than those removed from untreated controls in diestrus. Although the number of corpora lutea present in such ovaries was within the range (8–20) expected to be found after suppression of the estrous cycle, their histological appearance (fig. 1) indicated that they were retained from the last ovulation and that a new generation of corpora lutea was not formed. Five animals, which were in diestrus while receiving lactogen, came into estrus 2 days after the last injection and were autopsied at that time. These rats possessed ovaries containing old as well as recent corpora lutea and developing follicles (fig. 2), indicating that follicular development and function was held in abeyance during lactogen treatment.

Although the rat's uterus does not give a typical progestational response (23), the condition of the uterine epithelium and glands and the presence of vesicular nuclei in the stromal cells (24) suggested that the secretion from the lactogen-maintained corpora lutea had a progestational effect. Secretion of progestin by such corpora lutea was further indicated by the formation of spontaneous placentomata in the uteri of three of the animals treated with one of the lactogens (Schering). Two of these animals received a total of 133 I.U. and the third 160 I.U. over a period of 8 days and were killed on the 9th day. These placentomata were situated just above the cervix uteri; one site (containing large blood sinusoids) was found in the right uterine horn of each of the former two animals (fig. 3) and two sites, one in each horn, in the latter animal (fig. 4). Except as additional evidence that progestin was formed by these corpora lutea, no great significance can be attached to the presence of these placentomata, since they are known to occur spontaneously in a small percentage of both normal rats and rats under various experimental conditions.

Although the adrenals of the treated animals were histologically normal, they showed an average increase of 11.3 per cent[3] in weight over those of diestrous-normal controls, indicating the presence of a cortico-adrenotropic substance in both prolactin preparations. A splanchnomegalic effect of the lactogens was not determined since all of the viscera were not weighed.

Treated-adrenalectomized animals. To determine whether the secretion from lactogen-maintained corpora lutea would help prolong the survival time of adrenalectomized rats, 50 animals were bilaterally adrenalectomized; 20 were used as untreated controls and of the remaining 30, 15 were given Difco prolactin and the other 15 Schering prolactin. Each treated animal received an average of 20 (10–25) I.U., subcutaneously, daily (first injection given on the day of operation), during the period of survival or until the 15th postoperative day for those which survived longer. All the adrenalectomized animals were kept in a thermostatically controlled room and given the regular diet for our colony (Wayne Dog Blox) and tap water *ad libitum*. The procedure for making records and securing tissues for histological study was the same throughout the experimental period and at autopsy, as for the treated-normal rats referred to above.

Most of the treated-adrenalectomized animals showed the same type of estrus suppression as demonstrated by the treated-normal rats, differing in this respect from untreated-adrenalectomized animals which had completed 1 or 2 periods of estrus be-

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[2] As in the case of other pituitary preparations, individual animals may not respond (25) or may develop antihormones with long-term therapy (22).

[3] Increases or decreases in organ weights were calculated from the difference in the ratios of gland weight/body weight.

fore the terminal diestrus. All but 8 treated animals remained in diestrus during the period of survival.[2] Although the ovaries of the treated-adrenalectomized animals weighed 14 per cent[3] less than those of the untreated controls, due apparently to an early suppression of the estrous cycles with the formation of a smaller number of

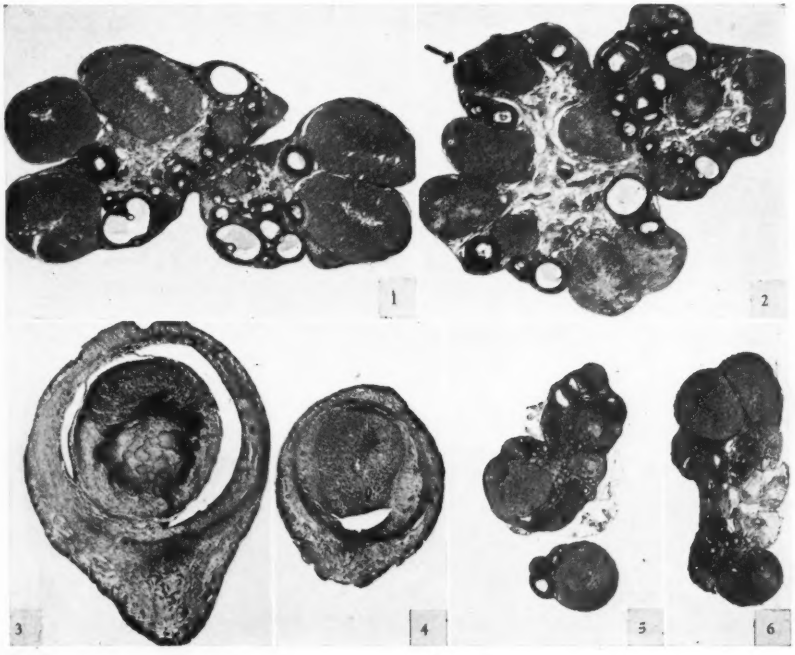

Fig. 1. OVARY, NORMAL ANIMAL TREATED with 20 I.U. of prolactin for 7 days, killed on 8th day. Maintained corpora lutea and maturing follicles are present in this ovary. Fig. 2. OVARY, NORMAL ANIMAL TREATED with 25 I.U. of prolactin for 10 days, killed on 12th day. Note arrow at point of rupture of follicle, structure of recently formed corpora lutea and developing follicles. This animal came into estrus on the 12th day.

Fig. 3. PLACENTOMA, NORMAL ANIMAL TREATED with 16.8 I.U. of prolactin for 8 days, killed on 9th day. Note large blood sinusoids in center of placentoma. Fig. 4. PLACENTOMA, NORMAL ANIMAL TREATED with 20 I.U. of prolactin for 8 days, killed on 9th day. Fig. 5. OVARY, ADRENALECTOMIZED ANIMAL TREATED with 25 I.U. of prolactin for 6 days, killed on 7th day. Compared with the ovary from the untreated control (fig. 6), there is better maintenance of the corpora lutea and development of the follicles in this ovary. Fig. 6. OVARY FROM UNTREATED ADRENALECTOMIZED animal killed on 7th postoperative day.

All photomicrographs of hematoxylin and eosin stained sections. ×10.

corpora lutea, there was less regression of the corpora lutea and better follicular development (fig. 5), than in those of their controls (fig. 6).

Even though both lactogens had no effect in maintaining postoperative body weight, the group treated with Difco prolactin but not those receiving the Schering prolactin, showed a significant increase in survival time as compared to that of the untreated controls (table 2). This difference in response obtained with the two lactogens was not due to any significant difference in the number or histological appearance of the corpora lutea in the two groups. Since prolactin is known to alter the metabolism of normal animals, and since the adrenalectomized rat is sensitive to

TABLE 2. ADRENALECTOMIZED ANIMALS TREATED WITH DAILY SUBCUTANEOUS PROLACTIN INJECTIONS

Number of Animals	Prolactin	Survival			P.E. Mean Difference
		Mean	P.E.	σ	
			days		
15	Difco	16.3	±.88	±5.04	±3.28
15	Schering	14.3	±.69	±4.01	±0.88
20	(controls)	13.2	±.55	±3.67	

$$\sigma\sqrt{\frac{\Sigma d^2}{n-1}}$$

metabolic changes, it seems possible that the Difco prolactin produced a deleterious effect on metabolism which nullified the beneficial action of increased luteal function in prolonging the survival time of the adrenalectomized rats. The limited survival period of both adrenalectomized groups, treated with lactogen, must have been due to the production of progestin in amounts insufficient for life maintenance over an extended period of time.

SUMMARY

Subcutaneous injections of lactogen daily for a period of 4 to 10 days to normal rats, caused maintenance of corpora lutea which apparently secreted progestin. Adrenalectomized rats, similarly treated from the day of operation until death or until the 15th day for those which survived longer, had a significantly prolonged survival time when treated with one lactogen (Difco) but not with the other (Schering) and showed better maintenance of follicles and corpora lutea than the untreated controls.

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STUDIES IN CORTICO-ADRENAL FUNCTION

I. SURVIVAL OF ADRENALECTOMIZED RATS

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UNTIL A FEW YEARS ago the question of the survival period of rats after bilateral adrenalectomy was the subject of dispute. The state of confusion is now being gradually resolved by a clearer understanding of the many factors which bear on this problem.

Death, the end-point of the survival period after adrenalectomy, appears at first sight to be simple and conclusive, and as a consequence unlikely to lead to ambiguity. This is by no means the case. In adrenalectomized animals death may ensue as a result of causes remote from, or seemingly unconnected with, adrenal insufficiency. Post-operative shock is the most important of these causes. It is intensified by the absence of adrenals, but operates even when the adrenals are not excised. It is in this connection that strain differences in animals may affect the survival period (1, 2, 3) quite apart from any question of inadequacy of cortical hormones. Greater susceptibility to traumatic shock may be the cause of the slightly shorter survival period in younger, as compared with older, rats. Factors such as the care and condition of the animals prior to operation (4, 5) influence to a greater extent the degree of shock than the hormonal requirements of the animal after a successful operation.

The fact that the animal appears to recover speedily after operation does not necessarily imply that it is free from shock since the adrenalectomized rat apparently has sufficient hormone in circulation for immediate needs. When the reserves of hormone are exhausted, only then do the effects of trauma manifest themselves and it is here that dietetic inadequacies (6–9), fluctuations in temperature (10–13), draughts and sub-optimal living conditions (7) exert their profound effects.

In certain strains of rats, however, it has been shown that the presence and distribution of accessory cortical tissue make it impossible to remove all sources of endogenous adrenal hormones (3). In these instances the proportion of indefinite survivals is greatly increased. Fortunately in our strain of rat, adrenal rests are very rarely found.

The survival period of adrenalectomized rats is of the utmost importance, as upon this depends the interpretation, not only of the effectiveness or otherwise of cortical extracts, but also of the nature of the bodily states in these animals. For this reason, therefore, at the commencement of our investigations into cortico-adrenal function it was necessary to ascertain the length of the survival period of our strain of white rat, bearing in mind the desirability of being able to recognize those deaths due to post-operative shock.

It is the purpose of this paper therefore to examine the survival period of rats after adrenalectomy, to ascertain whether age in any way prolongs or reduces this

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survival period and whether there is any advantage in using young or old rats in investigations on cortico-adrenal activity.

MATERIAL AND METHODS

General considerations. Throughout our experiments we have used the Lister Institute strain of albino rats, obtained originally from the colony of the Veterinary Research Laboratory, Onderstepoort. All of the rats used were bred under carefully controlled conditions and kept at a constant temperature (75° F.), away from draughts.

Unweaned rats (10–30 gm.) remained with their mothers after adrenalectomy and consequently received only mother's milk. The older rats were all kept on a standard diet fully adequate to promote normal growth and development. This consisted of: yellow corn meal 43.8 per cent; peanut meal 6.2 per cent; meat meal (80% protein) 6.2 per cent; Grand Sussex oats 15.5 per cent; Grand barley meal 8.2 per cent; copra meal 5.2 per cent; limestone 1.0 per cent; cod liver oil 1.0 per cent.

In order to ensure a satisfactory comparison, litter mates were distributed in different experimental groups.

Operative procedure. The operation is performed in one stage. Animals are anaesthetized in a closed vessel containing ether. If the correct amount of anaesthetic is used, it is possible to perform the whole operation without requiring any further ether.

The animal is placed on its abdomen with fore and hind limbs well extended. In this position the spine tends to arch and we have discovered that this arching is a useful landmark in determining the position of the skin incision. Immediately over the convexity of the spine the skin is pinched up and a small longitudinal incision about .5 in. long is made in the midline, just over the spine. The underlying connective tissue is separated from the skin with a sharp scissors. This is a necessary procedure as otherwise it is not possible to approach easily both sides of the animal.

The animal is turned slightly on its left side. The skin is retracted and the lumbo-dorsal veins are identified on the right side. The exact position of the incision through muscle is determined exclusively by the age of the rat. A small snick is made with a sharp pointed scissors and thereafter the closed scissors is inserted through the wound and the blades are opened to provide a wound one-quarter of an inch in length running parallel with the lower border of the last rib. If the muscle incision is made in the correct place then the adrenal may be seen lying in the depth of the wound. By gently grasping the surrounding fat the suprarenal bulges through the wound and can be easily excised. Without suturing the muscle wound the animal is turned slightly on to its right side and again the lumbo-dorsal veins are identified by retracting the skin edge to the left. The muscle layers are divided in the same manner as described above. The left suprarenal, in our experience, is removed more easily than the right.

The skin wound is closed with two or three interrupted sutures.

It is not possible to provide an easy rule for determining the position of the incision through the muscle. This can only be learned by experience. Our technique has reached such a stage of perfection that with proper team work we are able to adrenalectomize 20 animals in one hour.

RESULTS

Post-operative shock. The first series of adrenalectomies were performed on the heavier animals weighing between 40 and 120 gm. Of a total of 137 animals only 2 died within the first 24 hours of the operation. This number was so small that it did not affect the main issue. On the other hand, when we later removed the adrenals from rats weighing between 10 and 30 gm. no less than 18 of 68 died within the first 2 days after the operation. Firor and Grollman (14) found that all of their rats (6 in all, weighing less than 50 gm.) died on the first day after the operation. Even in older rats (50 to 100 gm.) these investigators lost 14 of 52 rats on the first day of the opera-

tion, but in rats over 100 gm. only 7 of 101 died on the first day. From their study Firor and Grollman concluded that 'it will be seen that the age of the rat is the predominant factor in determining the survival period. With increasing size (i.e., age) the survival period increases, rats weighing less than 50 gm. surviving on an average 1 day, those weighing over 200 gm. surviving on an average 9 days.'

In view of the fact that in our experiments as well as in those of Firor and Grollman a large number of smaller rats died on the first or second day after adrenalectomy, we felt it desirable to discover whether these deaths were due to post-operative shock.

Accordingly we took 13 young rats weighing between 10 and 20 gm. and subjected them to the same procedures as those animals from which the adrenals were removed, except that a small piece of peri-renal fat was excised instead of the adrenals.

TABLE 1. EFFECT OF AGE UPON THE DURATION OF LIFE OF ADRENALECTOMIZED RATS

Group	Total in Group	Body-Weight Range (gm)	Number of Rats Dying on Each Day After Operation													Deaths From Shock	Indefinite Survivals	Total Used for Calculation	Mean Survival Time (days)
			Day																
			1	2	3	4	5	6	7	8	9	10	11	12	13	14				
A	32	10–20	2	8		8	5	2	2		2		1				10	2	20	5.6±0.44[1]
B	36	21–30		8	2	1	3	1	4	5	4	2	2	1		1	8	2	26	7.7±0.55
C	37	31–40		2	1	4	9	8	3	5	4	1					3	0	35	6.3±0.30
D	34	41–50			2	3	8	9	10				1				0	1	33	5.8±0.26
E	35	51–60		1	4	3	7	8	10	2							1	0	34	5.7±0.26
F	35	61–80		1		6	13	4	6	2		2			1		1	0	34	6.0±0.34
G	33	100–120				1		3	10	5	5	3	1	1	2	1	0	1	32	8.4±0.40

[1] Standard error of the mean $\sigma_m = \frac{\sigma}{\sqrt{n}}$

Three of the 13 rats died within the first 48 hours. The remainder survived to maturity. This experiment indicates that it is possible to obtain a 23 per cent death rate due to trauma from the operation alone.

On comparing these findings with those in adrenalectomized animals (table 1) it is at once evident that in the group weighing 10 to 20 gm. 30 per cent died within the first 2 days and in the group weighing 21 to 30 gm., 22 per cent died. The 10 to 20 gm. group, being very young animals, could not stand the operation as well as the older rats; but there seems little doubt that in all young rats weighing up to 30 gm., the shock of the operation is sufficient to produce a death rate of 20 to 30 per cent.

The early stages in the development of the technique for adrenalectomy show very definitely that a large number of animals died within the first or second day of the operation. This is shown in the work of Brown-Séquard (15) who removed *only one* adrenal from each of 16 rabbits, 5 guinea-pigs, 2 cats and 2 dogs and in every case the animal was dead in 23 to 24 hours.

Many workers subsequently obtained similar results. Strehl and Weiss (16) found rats survived 15 to 19 hours, while cats, rabbits, guinea-pigs, mice and frogs all succumbed to bilateral adrenalectomy in 4 to 47 hours. Cristiani and Cristiani (17) state, 'Pour ce qui est de l'extirpation totale, nos résultats sont de plus uniformes et concordent en tout avec ceux de la plupart des expérimentateurs: la mort en est toujours la conséquence, mort qui survient d'un temps assez fixe, de 10 heures à 20 heures aprés l'opération.' In 1910 Schwarz (18) reported that his rats died within a few hours after operation. Even as late as 1923 Lewis (19) found death occurred in 400 rats generally in the first 48 hours after operation, exceptionally 3 or 4 days later.

Rogoff and DeNecker (20) were the first to point out that in Lewis' animals death might be ascribed to surgical causes and not to adrenal insufficiency.

Firor and Grollman (14) on the evidence presented, were unjustified in claiming that young rats died sooner after adrenalectomy than older ones. It seems more likely that all of their rats under 50 gm. in weight died as the result of shock; half of the number between 50 and 75 gm. and one-fifth of their 76 to 100-gm. group also died within the first day after adrenalectomy. As will be shown later, the general con-

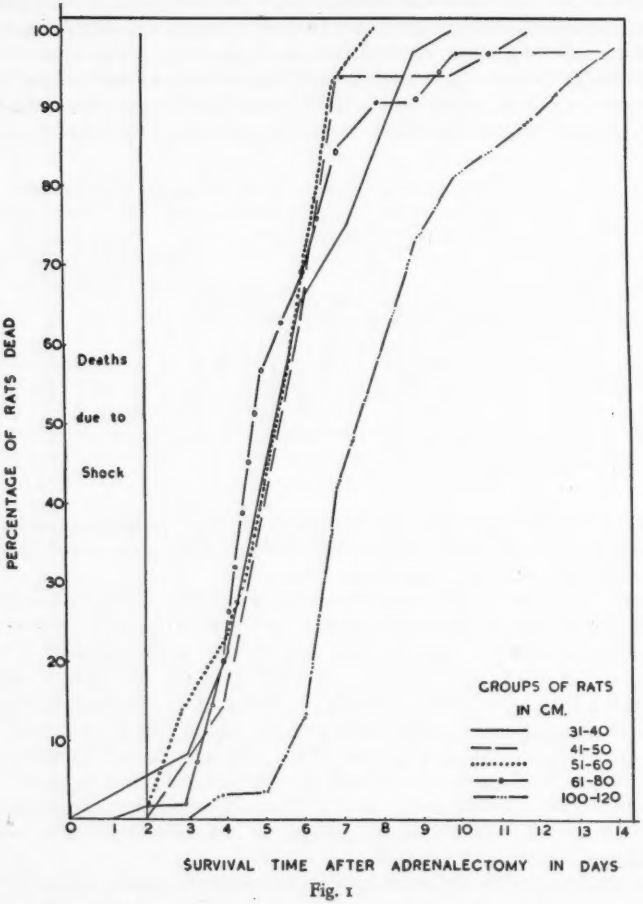

Fig. 1

clusions of Firor and Grollman as to the effect of age on the survival period are not supported by our own findings.

For these several reasons, therefore, we are led to conclude that death supervening within the first 2 days after operation cannot be reliably regarded as resulting directly from the removal of the adrenals, but that very probably it is caused by the operative trauma and the subsequent development of fatal shock.

Effect of age on survival. If we disregard those rats dying within the first two days after operation we see from table 1 that with the exception of the 100 to 200-gm. group the average survival period of rats weighing from 10 to 80 gm. is more or less

the same. The differences in the mean survival time in the various age groups is so slight as to be statistically insignificant (table 2). On the other hand the average survival period of the 100 to 120-gm. group was sufficiently longer to be statistically significant.

This is clearly shown in figure 1. Here we have plotted the total percentage of

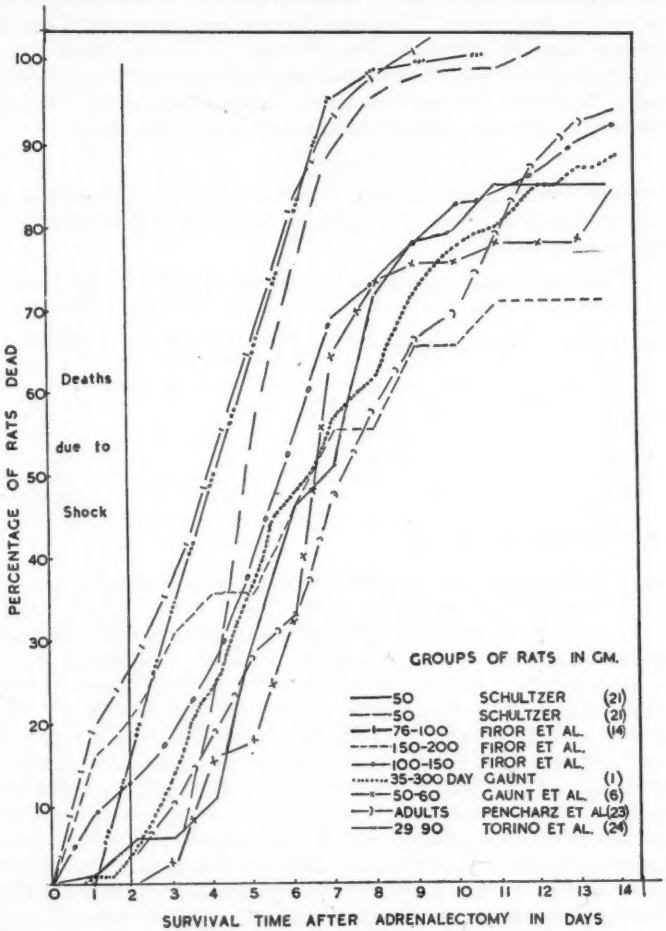

Fig. 2

deaths against the number of days of survival after operation. In view of what has been said above with regard to shock, the groups weighing 10 to 20 and 21 to 30 gm. have been omitted from figure 1. For purposes of comparison, some suitable data from the literature have been grouped in figure 2. The essential similarity of these results with ours is very striking. As compared with the findings of other investigators who used animals of a similar or greater weight these figures show that, in our series, a lower mortality rate exists during the first few days after operation.

An illuminating illustration of the manner in which these early deaths occur is provided by the two sets of data submitted by Schultzer (21) and represented in figure 2. In his first series of 50-gm. rats Schultzer found 14 per cent survived indefinitely. With improvement in operative technique the percentage of indefinite survivals was reduced to zero. All of his animals died within 12 days. Simultaneously with the improvement of technique, it is noteworthy that none of Schultzer's animals died within the first 2 days. This in itself further emphasizes the claims we have already made that early deaths are probably caused by operative shock.

Survival and adrenal rests. When animals survived for longer than 11 days a careful histological examination was made for residual adrenal tissue or rests. In 3 of 6 rats surviving indefinitely some adrenal tissue was identified histologically.

DISCUSSION

In table 3 are summarized the survival rates of adrenalectomized rats as reported during the past 10 years. They are classified in order of the increasing size of rat and our own values are included.

TABLE 2. STATISTICAL COMPARISON OF SURVIVAL TIME OF RATS IN VARIOUS GROUPS

Groups Compared	Mean Difference	Mean Difference / S.E. of Difference[1]	Significance of Difference
A—D	0.2	0.76	None
B—D	1.9	5.21	?
C—D	0.5	3.25	None
E—D	0.1	0.74	None
F—D	0.2	1.11	None
G—D	2.6	11.66	Significant

[1] S.E. of difference $=\sqrt{(\sigma_{m_1})^2+(\sigma_{m_2})^2}$.

Where an abnormally high proportion of indefinite survivals has been reported such results have not been included in table 3 as it is irrelevant to our present discussion. In a number of cases cited in the table survival rates above the average are accompanied by a high proportion of indefinite survivals. This is even more true in many series which have not been quoted here. For example, Gaunt (1) reported a survival period of 7 days in four colonies of adrenalectomized rats; 5 per cent of the animals lived a month or longer. In a fifth colony the life-span was 14.4 days; here however 45 per cent of the animals survived operation by more than a month. Swann (9) found mortalities of 95 per cent on diets associated with a life span of about 5 days after operation. On a diet containing bread the median life span was about 9 days and the mortality 72 per cent. Cleghorn and his collaborators (2) found that rats of the Connecticut Laboratory strain survived much longer than other strains. But whereas in the other strains the percentage of extended survivals was 0 to 2.9, in the Connecticut strain it was 10 and 19 per cent. In view of these considerations it is regrettable that some authors (14) should fail to make clear the proportion of indefinite survivals occurring in each group studied by them.

It is noteworthy in table 3 that, despite the great variety of experimental procedures employed by the different authors, 19 of a total of 22 survival rates lie between 5 and 9 days. This indicates a fair measure of agreement and raises the question: in view of the multiplicity of factors influencing the life-span of adrenalectomized rats, to what extent can it be legitimately regarded as due to adrenal insufficiency?

In figure 2 we have shown graphically the results of 6 investigators, all employing strains of rats with similar proportions of indefinite survivals. It will be seen that they

fall roughly into two groups depending on whether or not the mortality was 100 per cent. That the difference between these groups is not due to the strain of rat or to the same strain reared in different colonies, or to diet or any environmental factor is shown by the results of Schultzer (21) previously mentioned. Using his original operative procedure, he obtained results which definitely fall into the lower group. As a result of improvement in technique, all other factors remaining unaltered, his results now fall into the upper group. While it is not our intention to assert that the two groups of curves are completely distinct, a sufficient difference between them does exist to indicate the influence which the operation *itself* can have on results.

It cannot be doubted that a balanced diet is an essential factor in such studies as

TABLE 3. SURVIVAL OF RATS ADRENALECTOMIZED AT VARIOUS AGES

Age, days	Weight, gm.	Total Number of Rats	Average[1] Survival, days	Range, days	Indefinite Survivals,[2] %	Authors
	35–60	78	5.2	–11	5.1	Cleghorn et al. (2)
	31–60	103	5.9–0.3	3–11	1.0	Present authors
	37–50	17	6.7	5–10		Weiser and Norris (12)
	40–50	40	12.0±0.1	1–30	20.0	Emery and Schwabe (22)
	50–60	10	7.4	–12		Boilot and Choay (25)
	50–60	192	6.9	–15	2.5	Cartland et al. (26)
26–30		22	4.6	3– 8		Freed et al. (27)
28–35	50	58	6.6	1–12	14.0	Schultzer (21)
28–35	50	77	5.7	3–12		Schultzer (21)
30–32		500	8.5	5–19	4.0	Blanchard et al. (28)
30		72	8.2±0.8	3–30	5.6	Gaunt et al. (3)
30		56	8.1	4–16	9.1	Marrazzi and Gaunt (29)
	51–75	43	8.1	5–12		Weiser and Norris (12)
	60–80	15	5.5			Cleghorn et al. (2)
	60–80	35	6.0±0.3	4–13		Present authors
	80–100 (males)	11	7.3			Cleghorn et al. (2)
	110–120	33	8.4±0.4	4–14	3.0	Present authors
	100–183	15	9.7			Cleghorn et al. (2)
35–100		130	7.0	1–21	5.0	Gaunt (1)
	Over 100	60	8.2	2–18		Pencharz et al. (23)
Adults		24	17.8±2.5	–30	25.0	Gaunt et al. (6)
	<50–>200	180	5.3	1–21	12.2	Firor and Grollman (14)

[1] Excludes those classed as indefinite survivals.
[2] Those animals surviving beyond the last day of the range indicated.

the present. That the importance of diet should not be over emphasized is shown by the results of our groups of rats weighing 10 to 20 and 21 to 30 gm. These animals were all breast-fed, the mother being treated in the same manner as the older groups. Despite the complications of post-operative shock in these very young rats the influence of breast feeding as opposed to the ordinary diet is not reflected in the life spans of these groups.

It is our conclusion that once the strain of rat, its diet and environmental conditions have been carefully standardized, only operative technique is of importance, the age of the rat, within the limits of weight 30 to 80 gm. being without influence.

From the foregoing considerations it is evident that rats over the weight of 30 gm. are most suitable for experiments on cortico-adrenal activity. It is a well-established fact that adrenalectomy inhibits the growth of young rats. This is a matter of great importance because it is possible to follow the day to day progress of the experiment by means of the weight curve. On the other hand, mature animals have a fairly constant weight and the only gross indication of the bodily state is the survival period or any obvious changes which are associated with cortical insufficiency.

It seems to us that for assay purposes, rats weighing between 30 and 60 gm. are most suitable. The average survival range is amazingly constant, deaths from operative shock are infrequent, extended survivals rare, and the weight curve of each animal is an accurate representation of the effectiveness or otherwise of the treatment.

SUMMARY

Bilateral adrenalectomy was performed on a total of 248 rats ranging in weight from 10 to 120 gm. These were divided into 7 weight groups. The mean survival period of the groups in the weight range of 31 to 80 gm. was, in general, of the same duration, viz., 5.7 to 6.3 days. The 100 to 120-gm. group of rats had a slightly longer life span of 8.4 days.

Death during the first two days after operation was ascribed to shock. In the weight groups of 10 to 20 gm. and of 21 to 30 gm. the total number of animals dying from shock was greater than in all the other groups combined. This fact renders such animals unsuitable for experiments involving the extirpation of the adrenals.

The frequency distribution of the life span of animals in the groups of 31 to 80 gm. is such that these appear to be favorable for studies in cortical efficiency. From consideration of other factors cited, rats weighing between 30 and 60 gm. are most suitable.

Provided that the diet supplied is adequately balanced, there does not appear to be any significant difference in survival time; breast-fed groups (10 to 30 gm.) and weaned animals fed independently on a mixed diet have more or less the same average life span.

Given a standardized strain of rat weighing between 30 and 80 gm. on a balanced diet and optimal environmental conditions, only operative technique can influence the constancy of the survival period.

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WORK PERFORMANCE OF NORMAL RATS UNDER CONDITIONS OF ANOXIA

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EARLIER STUDIES (1, 2, 3) indicated that exposure to anoxia was followed by striking changes in the adrenal cortex. Repeated, short exposures to reduced pressure (2, 4, 5) resulted in a marked increase in the size of the adrenal glands and a corresponding decrease in the size of the thymus (5). Studies on adrenalectomized rats (1, 4) and dogs (6) suggested that these animals were unable to survive exposures at a reduction in pressure which was well tolerated by normal animals. However, the altitude tolerance of adrenalectomized rats (4) could be greatly increased by appropriate treatment with adrenal cortex extract. Synthetic desoxycorticosterone acetate therapy also improved the altitude tolerance of adrenalectomized animals but this compound was less effective in this respect than adrenal cortex extract. Preliminary experiments on normal animals, however, failed to reveal any significant improvement in 'altitude-tolerance' in normal animals when they were treated with adrenal cortex extract or desoxycorticosterone acetate. In these earlier experiments the animals were exposed under resting conditions to reduced pressure or to nitrogen-oxygen mixtures. It appeared desirable to ascertain what effect adrenal cortical hormone therapy might exert on the *work performance* of normal animals exposed to reduced oxygen tension since such experimental conditions would approximate more closely actual flying conditions.

In the present report we wish to describe a method which appears to be useful in approaching the problem of studying controlled work performance under conditions of anoxia. To obviate certain technical difficulties the apparatus has been designed for use with oxygen-nitrogen mixtures rather than reduced pressures. Preliminary experiments on the effect of adrenal cortical extract therapy, synthetic desoxycorticosterone acetate and benzedrine sulfate therapy on the work performance of normal rats under conditions of reduced oxygen tension are also presented.

METHODS

The apparatus consists of a large metal air-tight chamber measuring 50 × 63 × 63 cm. (fig. 1) which contains an electric fan and two circular, transparent, motor driven cages. The latter revolve on a single axle at a rate of 14 revolutions per minute. The small cages are lined with cellulose acetate paper which permits visualization of the interior of the cages and presents a smooth surface which prevents the animals from holding on to the wire mesh of the cage. Cross strips of one-inch wide adhesive tape,

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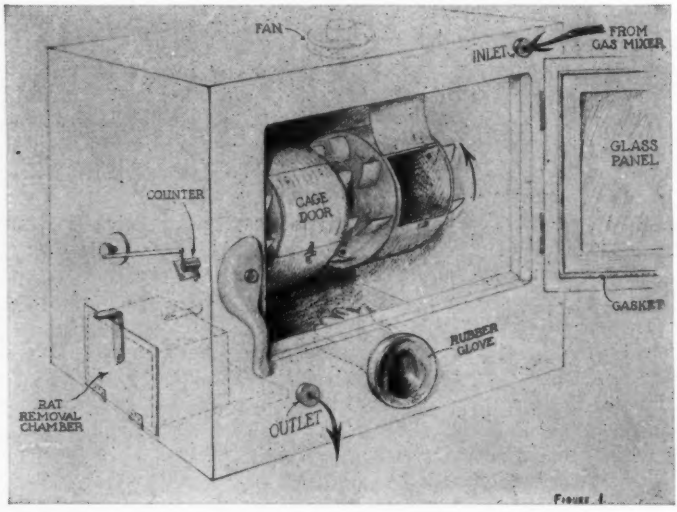
evenly spaced at intervals across the floor of the cage provide purchase for the animals during their running exercise performance. In addition, each cage contains two hurdles (2 cm. in height) which interfere very little with the exercise of the animals when they are fresh but which hasten exhaustion once fatigue begins. This results in a much more precise end-point. The cages are perforated by numerous apertures to permit free interchange of gases. Cardboard vanes further facilitate this process.

Each cage is equipped with a trap door which can be easily opened at any time, even while the cages are in motion. The trap door permits the removal of one exhausted animal from its cage without altering the rate of revolution of the adjoining cage which contains the control animal.

The large air-tight chamber is opened by a door especially fitted and lined with a rubber gasket which prevents air leakage. Illumination in the large chamber is ob-



tained by means of glass panels placed in the door and directly opposite in the rear wall of the chamber. Beneath the door there is a circular aperture closed by a long rubber glove which permits the contents of the chamber to be reached easily (fig. 1) without the necessity of opening the door. Thus manipulations inside the chamber can be carried on without altering the gas mixture.

A small metal 'air-lock' is attached to one side of the large chamber. This air-lock is fitted with two doors, one of which communicates with the interior of the large chamber and the other with the outside. During the course of an experiment the inner door of the air-lock is kept open. By means of this air-lock and the rubber glove it is possible to remove an exhausted animal from its rotating cage and transfer it to the outside without disturbing the composition of the gas mixture within a chamber or without stopping the second cage which contains a second animal. Thus, the work performance test can be continued on the second animal (control) until its end point of exhaustion is reached.

The animals used in these experiments were male, albino rats of the Sprague-Dawley strain, weighing approximately 150 gm. Training the animals to run in the

revolving cages prior to testing, improved the reproducibility of their performance. The training program consisted of a run on each of three successive days at atmospheric oxygen tension. This was followed by a run on each of three successive days under reduced oxygen tension (6.8 per cent oxygen; 93.2 per cent nitrogen). Training runs under anoxia were carried out until the animal was completely exhausted. Following the 6-day training period, control runs were then made at 4-day intervals to establish the control baseline of the animal's performance before treatment. When the second test varied from the first test by more than 10 per cent, additional control performance tests were made. Under these circumstances an animal was considered to be satisfactory when the deviation from the mean and subsequent tests was less than 20 per cent.

For each test, animals were paired, fasted for 18 hours and then forced to run in the revolving cages until completely exhausted. Experiments were carried out in an atmosphere of 6.8 per cent oxygen and 93.2 per cent nitrogen. The desired gas mixture was obtained by passing 230 liters of nitrogen into the large chamber at a rate of 20 liters per minute. During this 'flushing' process the fan was turned on and the trap doors of the cages were opened to insure adequate ventilation of the interior of the cages. Maintenance of the desired nitrogen-oxygen gas mixture was obtained thereafter by connecting the chamber to an automatic gas mixing apparatus (7) designed to deliver a mixture of nitrogen and oxygen (93.2 per cent nitrogen; 6.8 per cent oxygen) at a rate of 9 liters per minute. Samples of gas were removed from the chamber for analysis at the end of the flushing period and at appropriate intervals during the experiment. At the end of the flushing period the cages were set in motion and the animals were forced to run at a constant speed until exhausted. The end point of performance was taken as the time when the animal was no longer able to run over the hurdles but rolled helplessly on the floor of the cage. To insure greater accuracy the animal was allowed to roll helplessly on the floor of the cage for a period of exactly 2 minutes to ensure complete exhaustion; this latter 2-minute period was subtracted from the total running time to obtain the actual running time.

Testing two animals simultaneously permitted one member of the pair to act as a control for variations in temperature, slight variations in oxygen tension as well as the effect of repeated exposures. The animals usually recovered rapidly following removal from the chamber and could have been used again for subsequent tests after a period of 4 to 5 hours. In these experiments, however, 4 days was always allowed to elapse between the experiments on any individual animal.

OBSERVATIONS

Selection of animals for work performance tests. In these experiments only those animals were selected for study which on successive tests showed a reasonable degree of consistency in work performance. Animals whose reproducibility on the second control run varied from the first run by less than 10 per cent were considered satisfactory (table 1). When the second test varied from the first by more than 10 per cent, additional control performances were obtained. Under these circumstances an animal was considered to be satisfactory if the deviation from the mean in subsequent tests was less than 20 per cent. On the basis of this type of selection approximately 25 per cent of the animals were found to be unsatisfactory and were rejected.

During experiments each treated animal was paired and run with a suitable untreated control. These pairs of animals were kept in the same cage, fed the same diet, and were always tested simultaneously. By this method it was possible to control the effect of extraneous factors such as variations in room temperature and humidity, slight variations in the composition of the oxygen-nitrogen mixture supplied to the

chamber, as well as the effect of the repeated tests which were run during the preliminary training period (see methods).

Occasionally an animal was encountered whose performance exceeded by 200 to 300 per cent, that of the mean value of the other normal animals. Such an exceptional performance in the presence of such a marked reduction in oxygen tension was of great interest. The mechanism by which these few animals were able to perform so successfully is not known. An attempt is being made to breed these animals in the hope that a larger group of exceptional performers may be obtained for future study.

Effect of adrenal cortex extract therapy. The effect of adrenal cortex extract (Kendall) therapy on the work performance of normal rats under conditions of anoxia was studied in a group of 9 animals. After the consistency of their performance had been established (3 control periods), each animal was given 2 cc. of adrenal cortex

TABLE 1. DETERMINATION OF SUITABILITY OF ANIMALS FOR WORK PERFORMANCE TESTS UNDER CONDITIONS OF ANOXIA

Animal	Performance, minutes			Maximum Per cent Deviation from Mean	Comment
	First	Second	Third		
1	9.5	9.0		<5	Satisfactory
2	12.0	14.5		9	Satisfactory
3	17.5	14.5		8	Satisfactory
4	14.5	14.0		<5	Satisfactory
5	12.5	9.5	10.5	16	Satisfactory
6	28.5	23.5	30.5	14	Satisfactory
7	10.5	20.0		32	Rejected
8	9.5	12.5	16.0	25	Rejected

extract subcutaneously one hour before testing. At the same time 4 of the control animals were given an equal quantity of normal saline solution and the skin of the 5 remaining control animals was pierced with a needle but no solution was administered. Since there appeared to be no difference in the performance of the two control groups of animals the observations on these two groups have been combined in comparing their performance with that of the extract-treated normal rats (fig. 2).

It was noted that the extract treated animals had an average performance of 15.7, 14.6 and 13.3 minutes, respectively, on the first, second and third control performances. Following treatment with adrenal cortical extract, the average performance for the group was 16.5 minutes. This represents a 13 per cent increase in performance over the average performance of the three control periods. For comparison it is interesting to note that the three preliminary control tests on the animals that did not receive extract were 15.6, 15.1 and 13.3 minutes, respectively. The value for the fourth control period which corresponded to the treatment period in the extract-treated animals (fig. 2) was 17.8 minutes. This performance represents an increase of 19 per cent over the average of three previous tests of the control group. The increase which was noted in the final performance of the control group (saline-treated and needle-injected) was actually greater than that which was observed in the extract-treated group. From these preliminary experiments it is evident that 2 cc. of adrenal cortex extract treatment given 1 hour before the work test had no specific effect on the performance of normal animals.

The failure of adrenal cortical extract to improve the work performance of normal rats under these conditions has since been confirmed in another series of experiments (8 animals) in which larger doses of the hormone (total of 11 cc.) were given in divided doses for a period of 12 hours preceding the test.

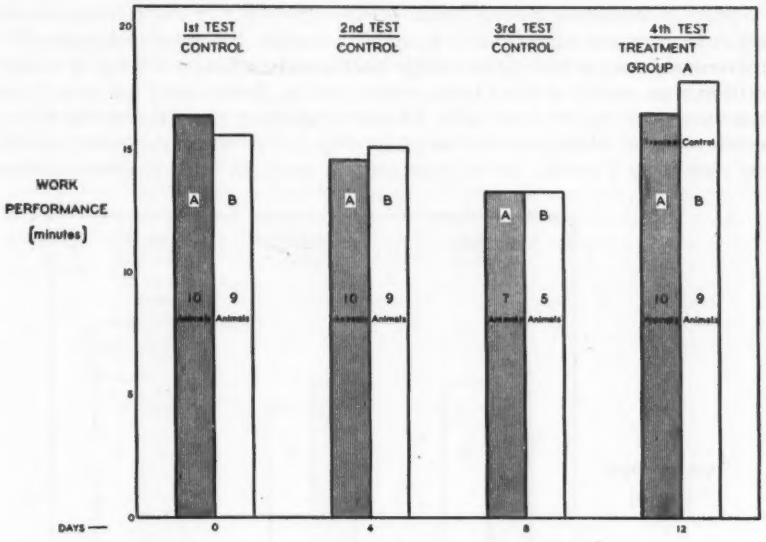


Fig. 2. Effect of adrenal cortex extract therapy on work performance of normal rats under conditions of reduced oxygen tension (6.8% oxygen; 93.2% nitrogen).

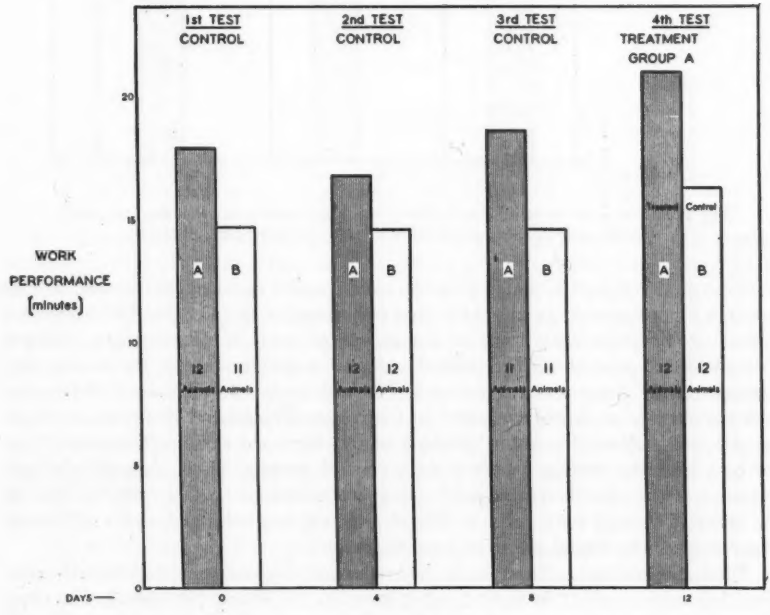


Fig. 3. Effect of desoxycorticosterone therapy on work performance of normal rats under conditions of reduced oxygen tension (6.8% oxygen; 93.2% nitrogen).

Effect of desoxycorticosterone acetate therapy. The effect of desoxycorticosterone acetate therapy was measured in a group of 12 animals. Following preliminary control runs, the animals were given a single intramuscular injection of 2 mg. of desoxycorticosterone acetate in oil 12 hours prior to testing. Both control and treated animals were given 0.9 per cent saline solution as drinking water during the fasting period (18 hours) which preceded the 4th test (fig. 3). The skin of the control animals was pierced by a needle, but no injection was made. In three successive control

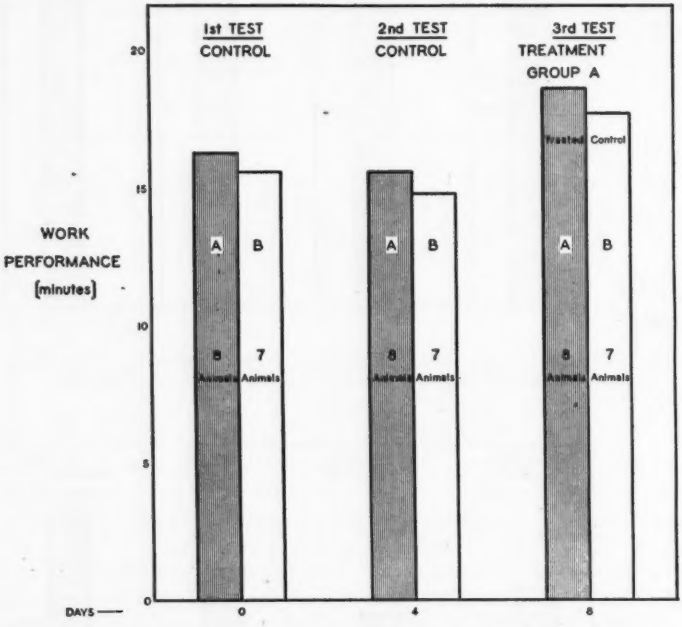


Fig. 4. Effect of benzedrine sulfate therapy on work performance of normal rats under conditions of reduced oxygen tension (6.8% oxygen; 93.2% nitrogen).

tests the group of animals which was selected to receive desoxycorticosterone acetate gave the following average values for their performances, 17.9, 16.8 and 18.6 minutes. Following treatment with 2 mg. of desoxycorticosterone acetate in oil, in addition to available 0.9 per cent sodium chloride solution as drinking water, the average performance of the group was 21.0 minutes. This represented an increase of 18 per cent over the average of the three control performances. The control group treated with 0.9 per cent sodium chloride in drinking water, increased their performance by 12 per cent over the average for their three control periods. Thus, although the performance of the animals treated with desoxycorticosterone acetate exceeded that of the controls treated with sodium chloride, we do not believe that the difference (6 per cent) can be considered to be a significant one.

Effect of benzedrine sulfate therapy. Following suitable control tests 8 animals were given benzedrine sulfate in doses ranging from .04 to .16 mg. per animal. The drug was administered subcutaneously in saline solution immediately prior to testing. All animals treated with benzedrine sulfate were considered as a single group (fig. 4) since

the change in performance following treatment appeared to be unaffected by the dose of benzedrine sulfate. At the time the treated animals were given benzedrine sulfate the skin of the control animals was pierced with a needle but no medication was injected.

In a second, confirmatory experiment, the effect of benzedrine sulfate (.04–.20 mg. per animal) was studied in 4 animals without simultaneous tests on control animals (fig. 5).

The observations in the first benzedrine sulfate experiment are summarized in figure 4. On two successive control tests the work performance averaged 16.3 and 15.6 minutes, respectively. Following treatment with benzedrine sulfate, the average

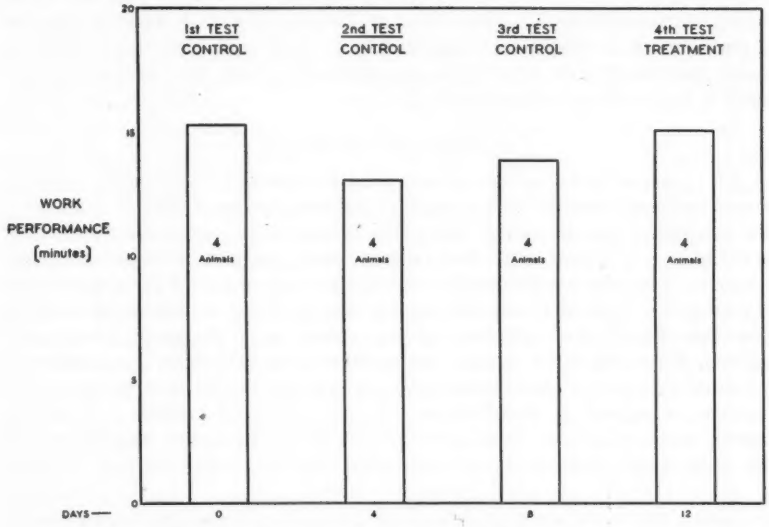


Fig. 5. Effect of benzedrine sulfate therapy on work performance of normal rats under conditions of reduced oxygen tension (6.8% oxygen; 93.2% nitrogen).

performance of the group increased to 18.6, an increase of 16 per cent over the average of the two control periods. However, in the control group of animals there was a similar increase (16 per cent) in the third test (fig. 4) over the average of the two preliminary control runs. Thus, it appeared that benzedrine sulfate treatment in a dosage of from .04 to .20 mg. per animal, administered immediately prior to work under conditions of anoxia, had no specific beneficial effect on the work performance of normal rats exposed to anoxia.

These observations are confirmed by a second group of experiments (fig. 5) in which the average performance after benzedrine sulfate was 15.1 minutes in comparison to average values of 15.3 and 13.1 minutes during two control tests.

DISCUSSION

The difficulties which are encountered in attempting to study work performance under conditions of anoxia are many. However, we believe that by the use of this technic, with care in training and selecting the animals, and with proper controls, it will be possible to study the effect of various pharmacological agents on the work performance of normal animals exposed to anoxia.

It should be pointed out that in these experiments we are dealing with a highly selected group of animals, each one of which had demonstrated its ability on several occasions to carry out this particular test satisfactorily. Poor performers would have been automatically excluded from these studies.

A number of other interesting problems are suggested by these studies. Foremost is the question of what peculiar faculties permit the exceptional animal to work for such extremely long periods under such adverse conditions? Unfortunately this problem cannot be investigated until larger numbers of these animals are available. It will also be of interest to determine whether these short, repeated tests (approximately 20 minutes) exert any effect on red blood cell formation, on adrenal and thymus size, as well as on the growth curve of young animals. The effect of repeated testing has not been studied on the ultimate work performance of animals. From the response of some animals it appears that improvement in work performance may follow repeated tests. In all of the experiments reported in this study, the fourth run was superior to that of the second and third.

SUMMARY AND CONCLUSIONS

An apparatus was devised and a method standardized whereby the *work performance* of small animals could be studied under conditions of reduced oxygen tension (oxygen-nitrogen mixtures). Using this technic, preliminary studies were made on the effect of treatment with adrenal cortex extract, synthetic desoxycorticosterone acetate and benzedrine sulfate on the work performance of a carefully selected group of normal rats exposed to reduced oxygen tension. None of these substances improved significantly the performance of these animals under the specific conditions as outlined. The necessity for extreme care in arranging suitable control experiments is indicated. The unusual performance (200–300 per cent greater than the mean) of a few animals suggests the possibility of attempting to breed a colony of 'super-performers' which could then be studied more intensively for factors which permitted such exceptional performance in an atmosphere of greatly reduced oxygen tension.

We are indebted to Dr. E. C. Kendall of the Mayo Clinic, Rochester, Minnesota, for the adrenal cortex extract which was used in these studies and to the Ciba Pharmaceutical Products, Inc., Summit, N. J., for the generous supply of desoxycorticosterone acetate.

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THYROTROPIC HORMONE ASSAY IN THE TADPOLE

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THE NEED FOR a sensitive, yet practicable, method for assay of thyrotropic hormone still exists in spite of the many and varied tests already reported by numerous investigators (1, 2). It is generally agreed, nevertheless, that the safest criterion of thyrotropic activity is one based on an observable stimulation of the thyroid gland in a test animal known to possess the gland in the 'resting' state. This condition exists normally in various animals such as the grass snake (3), 1-day-old chick (4, 5), or the young guinea pig (6, 7), whereas in others (rat, mouse) previous experimental treatment (hypophysectomy, special diets) must be administered in order to obtain a histologically inactive or resting thyroid gland (8, 9, 10). Although there has been some controversy regarding the degree of immaturity of the guinea pig necessary to show a resting gland, this animal has been used extensively for the bioassay of thyrotropin. The recent experiments of Smelser (4) and Bergman and Turner (5) have indicated, however, that the 1-day-old chick is more sensitive to thyrotropic hormone than the immature guinea pig. We have already shown (11) that total starvation of the frog tadpole prior to a critical developmental stage results in thyroid atrophy and a consequent metamorphic stasis which can be overcome with relatively small amounts of pituitary substance. The presence of an inactive thyroid gland in such 'stasis' animals, as well as the ease with which the metamorphic changes following hormone administration may be measured, made it desirable to test the suitability of the starved tadpole for thyrotropic hormone assay. Accordingly, these experiments will be concerned with a quantitative study of the thyroid and metamorphic response of the frog larva to thyrotropin.

MATERIALS AND METHODS

Rana pipiens larvae were obtained in large numbers by experimentally induced ovulation and fertilization (12). The hatched tadpoles were reared in tap water, maintained at laboratory temperature (22° C.) and were fed a diet consisting principally of ample quantities of boiled spinach. Space and volume factors, known to affect amphibian development, were minimized by keeping the developing larvae in aquaria, each containing 75 to 100 animals in approximately 15 liters of water. The water was renewed twice daily. Food was always available. When the stage of early hind-limb development had been reached, tadpoles with hind limbs 3–6 mm. in length were selected and divided at random into experimental and control groups. Experimental animals were then given intraperitoneal injections on alternate days, of 0.05 cc. of thyrotropic extract[1] in the following dilutions: 1:5, 1:50, 1:200, 1:800, and 1:3200.

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[1] We are indebted to Dr. Oliver Kamm, of Parke, Davis and Co., Detroit, Mich., for having supplied us with the thyrotropic hormone (Antuitrin T).

Control animals received 0.05 cc. injections of distilled water. Tadpoles in both groups were given no food throughout the entire experimental period.[2]

In the second series of experiments, 'stasis' (starved, non-metamorphosing) tadpoles which had been on total starvation for 16 to 18 days were subdivided into various groups. Animals in these groups were continued on the starvation regime, but were given on alternate days 0.05 cc. injections of the following: glucose (10%), glucosamine (10%), glutathione (1%), cysteine-hydrochloride (1%), sodium-diphosphate (1%), wheat germ oil, thyroxin (1:100,000), or thyrotropic extract. Corresponding groups of tadpoles which had been fed adequately to the same developmental stage up to the injection period were similarly treated. The development of the above animals was then compared to that of others fed normally prior to and during the experimental period.

Measurements of total length, body, and hind limb lengths were taken to the nearest millimeter by means of a ruled plate and dividers. The total weight of animals in each group was ascertained on a wet basis by collecting the tadpoles in a large net, placing the net on paper towelling for several minutes to drain off excess water, and removing the animals to a beaker which was then weighed on a trip balance accurate to 0.1 gm. The injections were made by means of a 1 cc. hypodermic syringe and a #27 gauge needle. Each animal, before being returned to water, was permitted to remain in the net for about one minute after injection. This prevented leakage of the injected material. Subsequent injections could usually be made at the same site. Weights and measurements of animals in all groups were taken at convenient intervals on days when no injections were given. The thyroid glands of representative animals in each group were prepared for microscopic examination by a decapitation method and histological procedure already described by one of us (13).

The thyrotropic extract was previously standardized against the immature guinea pig (175 to 225 gm. in weight) by determining the minimal amount of hormone needed to cause histological changes in the thyroid gland. The hormone was administered in 5 daily subcutaneous injections in the following amounts: 0.02, 0.01, 0.002, 0.001, and 0.00025 cc. On the 6th day the animals were killed, and the thyroid glands carefully removed and prepared for histological study. It was found by this procedure that the minimal effective dose of hormone which just induced structural changes in the gland in the guinea pig was attained with a total of 0.005 to 0.01 cc. of the extract.

RESULTS

Examination of the data (table 1) indicates that tadpoles normally fed to a stage of early hind-limb development (4–6 mm.) and then totally starved were accelerated in metamorphosis by several injections of 0.05 cc. of thyrotropic extract in dilutions as high as 1/3200. In contrast, water-injected controls remained in metamorphic stasis. The developmental advance in the hormone-treated animals was accompanied by a progressive loss of body weight which, by the third injection, exceeded that of the controls. In the higher concentrations used, 5 injections of the hormone totalling 0.00125 to 0.05 cc. were sufficient to cause a weight loss approximately 200 to 400 per cent greater than that shown by the water-injected controls. In animals receiving a total of 0.000078 cc. of the extract the weight loss exceeded that of the controls by at least 50 per cent.

The metamorphic changes in the hormone-treated animals stood in marked contrast to the arrested development shown by the controls. There was progressive ema-

[2] Actually, total starvation is only theoretically achieved since some absorption of food already in the digestive tract may occur. Cannibalism may also take place, although this occurred infrequently in our experiments.

TABLE 1. WEIGHT AND DEVELOPMENT CHANGES OF TOTALLY STARVED TADPOLES TREATED WITH THYROTROPIC EXTRACT

Dilution of Extract	Number of Animals	Initial Average Hind Limb, mm.	Mean Tadpole Weight gm.	% Weight Loss After			Total Amount of Extract Given in 5 Injections, cc.	Metamorphic Changes After 5 Injections	
				3 inject.	4 inject.	5 inject.		H. Limb, mm.	Body
1:5	30	5.8	1.69±0.02[1]	30.8±1.8[1]	40.5±2.2	58.0±2.3	0.05	12.4	Tail resorption
1:50	36	5.2	1.71±0.09	21.4±1.4	32.5±2.1	47.1±2.3	0.005	11.4	Fore limb emergence
1:200	43	5.3	1.76±0.13	21.2±1.5	25.9±1.5	37.8±1.0	0.00125	10.1	Just prior to fore limb emergence
1:800	27	5.1	1.67±0.07	15.1±2.1	18.0±1.3	23.4±1.6	0.000313	9.1	Constricton at fore limb region
1:3200	23	4.7	1.64±0.09	14.4±0.3	16.5±2.3	23.0±2.0	0.000078	8.8	Slight constriction
Water Control	36	5.1	1.75±0.07	8.6±1.3	13.2±2.2	15.4±3.6		7.1	None

[1] Mean± average deviation.

ciation, and changes of shape in the head and body, followed by constriction at the opercular region. A concomitant increase in hind-limb length occurred.

Almost invariably associated with this increase was a hyperemia of the hind limbs which appeared after the first few injections. The climax stages of metamorphosis (forelimb emergence, tail resorption) usually occurred with 4 or 5 injections of the extract in the higher concentrations, whereas these developmental events appeared after 7 to 9 injections when the greater dilutions were used. Aside from a relatively small increase in hind-limb length, the controls showed none of the somatic changes characteristic of either normal or accelerated metamorphosis (fig. 1).

In testing the metamorphic response of more advanced animals (12–18 mm. hind limb) to thyrotropic hormone it was found that usually 4 injections of the extract in dilutions of 1:200 enabled these tadpoles to attain the forelimb-emergence stage. The water-treated controls, however, unlike animals in early hind-limb development, continued in and completed their metamorphosis, although at a slower rate than the hormone-treated animals. The unsuitability of the advanced tadpole as a test object for thyrotropin will be discussed below.

A consideration of the data (table 2) reveals no marked difference in the rate of induced metamorphosis by thyrotropic hormone between animals starved (16–18 days) prior to hormone treatment and those adequately fed to approximately the same developmental stage. Although the hind limb extension was usually greater in the better nourished animals, the percentage of tadpoles extruding their forelimbs was about the same in both groups. Animals which were fed the normal laboratory diet throughout required slightly greater amounts of the hormone to attain the forelimb stage.

TABLE 2. COMPARISON OF METAMORPHIC ADVANCE OF STARVED AND FED LARVAE TO THYROTROPIC EXTRACT

Treatment	Number of Animals	Initial Mean Length Hind Limb, mm.	Days Starved Prior to Treatment	Number of Injections	Total Amount Extract Given	Metamorphic Advance	
						Mean Hind Limb Length, mm.	% Animals Showing Forelimbs
Thyrotropic Extract (Rowlands-Parkes)	30	2.2	16	7	0.7 mg.	8.1	73.3
	30	2.8	0	7	0.7 mg.	7.9	80.0
Antuitrin T., Dilution of 1:200	22	5.0	18	7	0.00175 cc.	13.0	58.3
	22	6.5	0	7	0.00175 cc.	18.2	68.4
	22	6.5	Fed throughout	10	0.0025 cc.	23.6	54.5

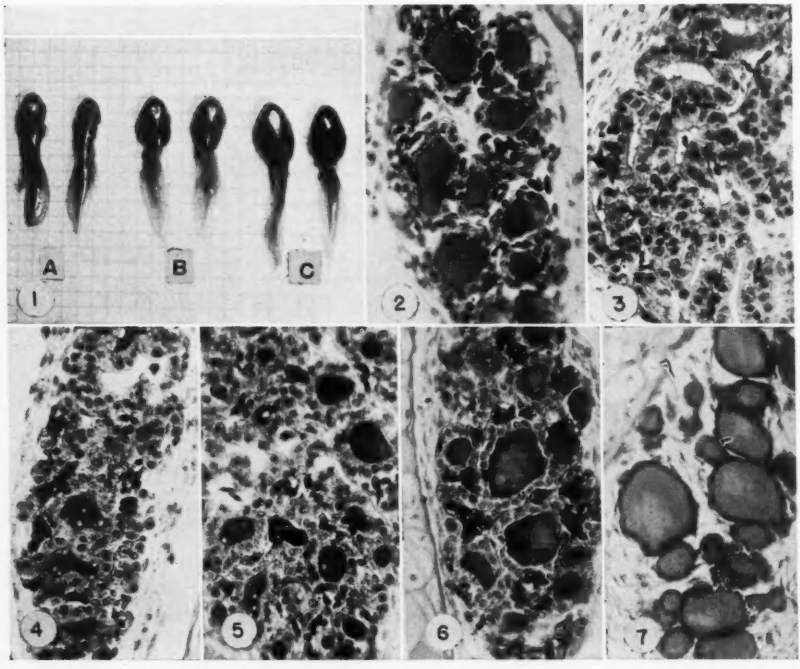

Fig. 1. PHOTOGRAPH (one-half actual size) of totally starved, experimental and control tadpoles illustrating the metamorphic changes induced by thyrotropic hormone. Note the smaller size, the extruded forelimbs and the adult pigmentation of animals (*A*) receiving a total of 0.0015 cc. of thyrotropin (6 inj.), and the body changes (*B*) with 0.000375 cc. of hormone, as compared with the non-metamorphosing water-injected controls (*C*). Fig. 2. THYROID GLAND of a stasis tadpole showing all the histological features of inactivity. Bouin; Masson. ×205. Fig. 3. ACTIVATED THYROID GLAND of a starved tadpole receiving a total of 0.05 cc. of thyrotropin (5 inj.). Note the marked hypertrophy and hyperplasia of the gland, and the practically complete colloid release. Bouin; Masson. ×205.

Fig. 4. DEGREE of activation in the thyroid of a starved animal given a total of 0.005 cc. of hormone (5 inj.). The stimulation is indicated by the tall columnar epithelium, and the collapsed follicles. Bouin; Masson. ×205. Fig. 5. ACTIVATED THYROID GLAND of a starved animal given a total of 0.003 cc. of thyrotropin (5 inj.). The heightened epithelium, as well as some liquefaction and vacuolation of the colloid indicate relatively moderate stimulation of the gland. Bouin; Masson. ×205. Fig. 6. THYROID GLAND of a starved tadpole receiving a total of 0.000078 cc. of thyrotropin (5 inj.). Note that aside from the greater vascularity, and slightly higher epithelium, the histology of the gland approaches that of the normal Bouin; Masson. ×205. Fig. 7. THYROID GLAND of a stasis tadpole (starved 16 days) made to metamorphose by thyroxin. All of the histological features of atrophy are present despite the advanced stage of the host (tail resorption). Zenker-formol; Masson. ×245.

Stasis tadpoles which were given either glucose, glucosamine, glutathione, cysteine, wheat germ oil, or inorganic phosphate made little or no progress in metamorphosis, their development being arrested like that of the water-injected controls. Thyroxin injections, on the other hand, caused resumption of metamorphosis in starved animals.

The microscopic picture of the thyroid glands in the starved non-metamorphosing control animals was one of inactivity. The follicles were distended with a dense, deeply staining, usually non-vacuolated colloid. The follicular epithelium was either squamous or low cuboidal (fig. 2). The nuclei tended to be pycnotic and hyperchromatic. The thyroid glands of all thyrotropic hormone-treated animals on the other

hand displayed structural alterations indicative of activation. The degree of stimulation of the glands was roughly proportional to the dose given (fig. 3, 4, 5, 6). A total of 0.05 or 0.005 cc. of hormone over a period of 5 injections caused marked hypertrophy and hyperplasia of the gland. The follicular epithelium was tall columnar, and secretion droplets were abundant at the apical region of the cell. The nuclei were rounded and enlarged. The lumina of the follicles were collapsed and colloid release was practically complete. There was correspondingly less activation with the lower dosages used. Five injections of the extract, totaling 0.001 or 0.0003 cc., caused liquefaction and vacuolization of the colloid and an increase in epithelial height. The structural changes which occurred in the thyroids of animals receiving a total of 0.000078 cc. of hormone were barely perceptible. Except for the follicular epithelium which was slightly higher than that in the control thyroids and a greater vascularity, the microscopic picture was practically the same as that of the control gland. This amount of hormone evidently approaches the minimal effective dose detectable histologically.

A comparison of the structure of activated thyroids in animals starved prior to hormone treatment with those of tadpoles fed to the same developmental stage revealed no marked difference in the degree of stimulation to equivalent amounts of thyrotropic hormone. Nor was there a great deal of difference in the degree of stimulation if thyrotropic hormone-treated animals were fed during the experimental period. These thyroids usually showed a larger amount of colloid than the activated glands of starved animals; nevertheless, the follicular epithelium in most follicles was equally as high. The morphological changes occurring in the activated thyroid were more discernible when it was compared with the resting gland of the starved non-metamorphosing animal than when compared with that of a fed animal which normally displays histological manifestations of varying secretory activity. This was not the case, however, with the thyroid of advanced tadpoles which, despite the starvation regime, continued apparently normal secretory activity. The activation changes in the thyroids of starved tadpoles appeared to be specific for material of hypophyseal origin, since neither glucose, glucosamine, glutathione, cysteine, phosphate, wheat germ oil, nor thyroxin stimulated, or even restored to normal, the atrophic thyroid picture of starvation (fig. 7).

On the basis of these results, a tadpole unit of thyrotropic hormone activity may be tentatively defined as the minimal amount of hormone which, when administered to the starved non-metamorphosing animal in 5 intraperitoneal injections on alternate days will result in a body-weight loss about 50 per cent greater than that of the water-injected controls. The weight loss in the hormone-treated animals must be accompanied by metamorphic changes and by stimulation of the thyroid gland as judged histologically. At least 15 animals in each group should be used in making the test. In terms of the unit so defined, it would appear that the minimal dose of thyrotropin required to activate the tadpole thyroid is approximately $\frac{1}{60}$ to $\frac{1}{120}$ of that necessary in the 200-gm. guinea pig.

DISCUSSION

Although it is well established that the accelerated metamorphosis of amphibian larvae with mammalian pituitary substance is due to a specific thyrotropic principle capable of activating the thyroid gland in normal or hypophysectomized animals (14, 15, 16), the quantitative aspects of this endocrine interaction have received scant attention. Atwell, (17) found that several injections of a thyrotropic extract (Collip)

were sufficient to stimulate markedly the thyroid gland and cause the resumption of metamorphosis in tadpoles hypophysectomized for months. Graded doses, however, were not tried. The sensitivity of the tadpole to thyrotropin was also suggested by the comparative experiments of Cuyler et al. (18) which indicated that relatively small amounts of thyrotropic extract (Rowlands and Parkes) could accelerate larval development. The histology of the thyroid, however, was not studied.

The results of our quantitative experiments demonstrate clearly that the tadpole does respond to extremely small amounts of thyrotropic hormone, and that the degree of response, moreover, can be measured by such criteria as the progressive body-weight loss and increase in hind-limb length or by direct histological examination of the thyroid gland. The criterion of loss of body weight by the hormone-treated animals, although necessarily measured on a wet basis, a method admittedly crude, is convenient and reliable for the bioassay of thyrotropin. The effect of the hormone, however, is more sensitively reflected by hind-limb extension and hyperplasia of the thyroid. We have found (unpublished experiments) that the thyrotropic principle in mammalian body fluids is detected sooner by these criteria than by the body-weight loss, although the latter becomes appreciable after continued injection.

We have previously submitted evidence (11) that starvation of the tadpole at the stage of early hind-limb development suppresses the thyrotropic function of the anterior hypophysis. Similar evidence for a derangement of thyroid-pituitary function in inanition is well known among mammals (19). In either instance, the structural alterations which appear in the thyroid gland after starvation are qualitatively similar to those appearing in the hypophysectomized animal. In fact, the production of metamorphic stasis and of a resting thyroid gland in the starved tadpole eliminates the necessity of such a specialized technique as hypophysectomy. It is important, however, that the organism be starved prior to a critical stage in development since animals adequately fed to this period continue their metamorphosis and give histological evidence of secretory activity in the thyroid despite the subsequent starvation. For this reason it appears less advantageous to employ the more advanced tadpole for the bioassay of thyrotropin.

It appears from histological examination that the thyroid gland of the starved tadpole is no more sensitive to thyrotropic hormone than that of the fed animal. This is contrary to the condition reported for the guinea pig (20) in which the thyroid of the chronically starved animal could be stimulated by one-tenth the minimal effective dose of thyrotropic hormone in the normal animal. The degree of thyroid stimulation induced by relatively high doses of thyrotropin is approximately the same in fed and starved tadpoles. The mild degree of activation after small doses of hormone are used is more obvious when it is compared with the starved control gland than when it is compared with the glands of fed animals since these normally exhibit varying degrees of histological activity. That the sensitivity of the starved thyroid is not appreciably increased by starvation is supported by the fact that tadpoles starved prior to hormone treatment metamorphose no more rapidly than animals which had been fed to the same developmental stage before injection was begun. The slightly slower rate of induced metamorphosis in tadpoles fed throughout the entire experimental period does not necessarily negate this view inasmuch as the increased growth due to feeding results in such additional complicating factors as greater protoplasmic mass to transform, or dilution of the administered hormone. In any event these differences in both rate of development and degree of thyroid activation are not sufficiently marked to accept at present the concept of increased sensitivity of the larval amphibian thyroid gland to thyrotropic hormone in starvation.

The hyperplastic changes seen in the thyroid glands of starved tadpoles apparently are specific for hypophyseal material. None of the various solutions or organic or inorganic substances tried in our experiments restored to normal the atrophic thyroid picture of starvation, although thyroxine, alone, was able to cause the resumption of metamorphosis in the starved animal. The ability of thyroxine (or thyroid gland substances) to accelerate amphibian metamorphosis by acting directly on the tissues of the organism is a well-known phenomenon in amphibian development (21).

Recent experiments have made it apparent that the chick reacts to thyrotropic hormone more readily than the guinea pig. Smelser (4) found that to cause a similar proportionate increase in thyroid weight, the 1-day-old chick required one-tenth the amount of thyrotropic extract necessary in the immature guinea pig. Although the minimal effective dose detectable histologically was not determined, the guinea pig thyroid required the use of 4 times the amount of hormone needed to produce the same degree of stimulation in the chick. Bergman and Turner (5) likewise found the chick more sensitive than the guinea pig on the basis of increased thyroid weight and consequently defined a male chick unit of thyrotropic activity equal to approximately one fourth that of the guinea pig. The micro-histometric methods of Rawson and Salter (22) have also shown that small doses of thyrotropic hormone (one-eighth Junkman-Schoeller unit) can significantly increase the epithelial height of the thyroid in the 1-day-old chick. A comparison of the response of the stasis tadpole with that of the chick or guinea pig makes it clear that the frog larva is an extremely sensitive organism for the bioassay of thyrotropin. Assuming the lowest concentration of extract used in our experiments as approximating the minimal effective dose, direct comparison to the 200-gm. guinea pig (against which the extract was standardized) demonstrates that the tadpole responds to 1/60 to 1/120 of the amount of thyrotropic hormone which can be detected histologically in the former animal.

Bergman and Turner (5) have already pointed out that the sensitivity of the chick and guinea pig to thyrotropin is of approximately the same order if body weight is taken into consideration. That this correlation is probably not fortuitous is strongly suggested by the fact that the smallest effective dose used in the tadpole, when equated to the body weight, produces a value similar to that obtained in the guinea pig. It seems significant that test objects from three vertebrate classes should show this positive correlation between thyroid response and body weight.

The factors which condition the thyroid response to thyrotropin are, at best, imperfectly understood. Thurston (23) has shown that there is a pronounced difference among various species in the reactivity of the thyroid gland to anterior pituitary extract. As measured by hyperthrophic changes, the order of increasing stimulation was found to be as follows: mouse, rat, rabbit, cat, pigeon, and guinea pig. Although there is some disagreement regarding this order (24), practically all investigators agree that the rat, in contrast to the guinea pig, is highly resistant to thyroid stimulation with thyrotropic hormone. There is some evidence that thyroid sensitivity may be inversely proportional to the thyrotropic content of the animal's own pituitary (25).

It is not unlikely that other factors, aside from some threshold or receptive property of the gland itself, influence the histological response of the thyroid gland to thyrotropic hormone. The correspondingly lesser amounts of hormone usually required in the smaller test object raise the possibility that the volume of vascular fluid may be involved. It might be anticipated thus, that in the smaller animal there would be less dilution of the administered hormone, and consequently a greater effective concentration available for the end organ. A second factor to be considered is the size of the thyroid gland itself. Evidence is accumulating from studies, both *in*

vivo and *in vitro* (26, 27), that thyroid tissue inactivates thyrotropic hormone. Accordingly, the smaller amount of thyroid tissue in the tadpole would conceivably result in less inactivation of the hormone, while producing its effect, than the comparatively large thyroids of other test objects. It would be generally expected, then, that the smaller the test organism the greater would be the histological response to some unit amount of thyrotropic principle. This hypothesis receives some support from bioassay experiments involving other tropic factors of the anterior hypophysis. In general, the immature animal seems more desirable for bioassay. Moon (28) employs the normal 21-day-old rat for the assay of corticotropin. More recently, Astwood and Tyslowitz (29) have used the 55-gm. hypophysectomized male rat to assay the corticotropic content of the pituitary of various mammals. In both instances a relatively small test object had been chosen. Bioassay of the gonadotropins, likewise, is usually made on the smaller organism, although it well may be argued here that the developmental state of the receptor organ is probably the major factor involved.

A further possibility which must be considered in attempting to explain the marked sensitivity of the tadpole to thyrotropin is that of low body temperature. The poikilothermic nature of the amphibian may possibly result in a lesser rate of destruction of such a thermo-labile hormone. In fact, a two-hour incubation of the hormone at 37° C. will result in some destruction as shown, when tested in tadpoles, by the slower rate of accelerated metamorphosis (unpublished experiments). The difference in sensitivity among homothermic animals, however, could not be thus explained. Indeed, until more is known of the metabolism and excretion of thyrotropic hormone in any organism, the factors proposed in this discussion must be considered speculative.

The high sensitivity of the tadpole, nevertheless, should prove to be of value in detecting the small amounts of thyrotropic hormone present under various physiological conditions in the mammalian organism. Practical considerations also contribute to the usefulness of this animal for thyrotropic hormone studies. Larvae can be secured at practically all times of the year. Hundreds of animals may be obtained from a single clutch, thus making possible a large scale experiment with genetically similar animals. Tadpoles, moreover, are easily maintained in the laboratory, require relatively little space, and tolerate experimental treatment well.

SUMMARY AND CONCLUSIONS

By the administration of graded doses of a thyrotropic extract to frog larvae, it was found that several intraperitoneal injections of the hormone, totalling 0.000078 to 0.05 cc., can induce metamorphosis in the stasis (starved, non-metamorphosing) tadpole. The metamorphic advance is characterized by *a*), progressive body-weight loss (50 to 400 per cent greater than that of the water-injected controls), *b*), by a concomitant increase in hind-limb length, and *c*), by activation of the thyroid gland. Histological examination of the thyroid in control animals invariably shows a resting gland against which the hyperplastic changes in hormone-treated animals are easily observed. The activation of the thyroid appears to be limited to material of pituitary origin. Stimulation of the gland in starved animals was not produced with glucose, glucosamine, gluthathione, cysteine, wheat germ oil, inorganic phosphate, or thyroxine. Thyroxine, however, induced metamorphosis. Total starvation (16–18 days) of tadpoles prior to hormone treatment does not alter appreciably the degree of thyroid activation nor the rate of accelerated metamorphosis induced by subsequent thyrotropin administration.

Direct comparison with the immature guinea pig indicates that the frog tadpole is a sensitive test object for thyrotropic hormone assay since the tadpole responds to

approximately 1/60 to 1/120 the amount of hormone which can be detected histologically in the guinea pig. A tadpole unit of thyrotropic activity is tentatively defined as the minimal amount of hormone which, when given to the totally starved non-metamorphosing animal in 5 intraperitoneal injections on alternate days, will stimulate the thyroid gland, increase the hind-limb length, and cause a body-weight loss 50 per cent greater than that in water-injected controls. It appears highly significant, however, that if body weight is taken into consideration, the sensitivity of the tadpole and of the guinea pig is of the same order of magnitude. The factors possibly involved in this correlation between thyroid response to thyrotropic hormone and body weight are discussed.

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INFLUENCE OF WEIGHT (AGE), DIET AND DOSAGE ON RESPONSE OF THYROID AND PARATHYROID GLANDS OF MALE GUINEA PIG TO POTASSIUM IODIDE; EFFECT OF THIS SUBSTANCE ON ADRENAL GLAND[1]

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THE RESPONSE OF the thyroid gland to the administration of potassium iodide has been a matter of controversy for many years. It has been maintained by Marine and his co-workers (1–3) that, when the iodine intake is lessened below the normal level, the thyroid gland tends to undergo hyperplasia, and, conversely, that the administration of iodine diminishes the activity of this gland, causing the production of hard colloid and low epithelium. Similarly, Levine, Remington and von Kolnitz (4) and also Chapman (5) have reported that hypertrophy of the thyroid of rats results from a low iodine intake. On the other hand, Loeb and his associates (6–13) have reported, in a large series of investigations, that the administration of KI primarily results in a stimulation of the thyroid gland, which is characterized by an increase in mitotic activity of the acinar epithelium associated with a softening of the colloid, an increase in the number of phagocytes in the colloid, and an increase in height of the cells lining the acini. This response occurs during the first three weeks of administration of this substance and is subsequently followed by an increased storage of colloid, which presses on the acinar epithelium and causes its flattening.

It is the purpose of the present investigations to present further evidence concerning the stimulating effect of KI on the thyroid gland and to define certain variable factors which may, to a certain extent, modify this response. In addition, further evidence is given relating to the parallelism in the response of the thyroid and parathyroid glands to the administration of KI, an observation which has recently been reported by Loeb and the author (14). The effect of this substance on mitotic activity of the adrenal cortex has also been studied.

MATERIALS AND METHODS

In the first experiment, a group of guinea pigs was divided into three groups according to weight (age): *Group I* consisted of 52 male guinea pigs which had just been weaned (approximately 2 weeks old) and which weighed between 100 and 115 gm. at the beginning of the experiment; *Group II* (46 guinea pigs) ranged between 180

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TABLE 1. INFLUENCE OF IODINE CONTENT OF DIET ON MITOTIC ACTIVITY IN THYROID AND PARATHYROID GLANDS OF MALE GUINEA PIGS OF VARIOUS WEIGHTS (AGES)

A, Diet Not Containing Chow						B, Diet Containing Chow Without Iodine						C, Diet Containing Chow With Iodine					
Number of guinea pigs	Average final weight, gm.	Days on diet	Average mitoses/ thyroid	Average parathyroid count		Number of guinea pigs	Average final weight, gm.	Days on diet	Average mitoses/ thyroid	Average parathyroid count		Number of guinea pigs	Average final weight, gm.	Days on diet	Average mitoses/ thyroid	Average parathyroid count	
				Mitoses/ 10,000 cells	Cells/ unit field					Mitoses/ 10,000 cells	Cells/ unit field					Mitoses/ 10,000 cells	Cells/ unit field
Group I. Initial weight 100–115 gm.																	
3	114	0	263	4.8	209[1]	3	114	0	263	4.8	209[1]	3	114	0	263	4.8	209[1]
4	135	5	89	1.4	205	2	150	5	45	1.5	223	2	155	5	180	1.4	218
3	140	10	50	0.8	215	2	215	10	93	1.7	180	4	214	10	189	2.8	190
2	138	15	50	0.9	198	2	263	15	88	1.0	200	2	248	15	198	3.1	198
4	197	20	97	1.1	180	2	280	20	50	1.5	185	5	244	20	303	4.5	178
4	220	30	565	7.0	200	2	330	30	853	6.7	173	5	270	30	331	5.4	140
2	210	40	140	3.4	180	2	340	40	195	4.0	200	3	310	40	985	6.8	153
		Average	186	2.6	197			Average	223	2.6	191			Average	316	4.3	173
Group II. Initial weight 180–200 gm																	
4	180	0	153	3.7	203[1]	4	180	0	153	3.7	203[1]	4	180	0	153	3.7	203[1]
2	215	5	160	2.1	190	2	218	5	120	1.0	200	2	210	5	130	1.9	180
2	245	10	200	1.0	200	2	230	10	110	0.5	210	2	270	10	160	2.1	171
2	240	15	130	1.1	195	4	237	15	136	1.8	188	6	268	15	210	2.6	176
2	255	25	100	1.4	185	4	288	25	141	1.6	183	6	285	25	254	2.4	175
2	274	40	70	0.6	210	4	339	40	90	0.6	173	4	384	40	156	1.1	190
		Average	132	1.2	196			Average	121	1.2	187			Average	199	2.6	179
Group III. Initial weight 390–420 gm.																	
4	418	10	47	0.8	198							4	408	10	65	0.3	197
4	447	25	52	0.3	205							4	435	25	40	0.5	202
4	495	40	45	0.7	188							4	504	40	20	0	220
		Average	48	0.6	197									Average	42	0.2	206

[1] Counts were made in these guinea pigs before the other animals of the respective groups were placed on the various diets.

TABLE 2. INFLUENCE OF WEIGHT (AGE) AND IODINE CONTENT OF DIET ON MITOTIC RESPONSE OF THE THYROID AND PARATHYROID GLANDS OF MALE GUINEA PIGS TO KI ADMINISTRATION

A, Diet Not Containing Chow						B, Diet Containing Chow Without Iodine						C, Diet Containing Chow With Iodine					
Number of guinea pigs	Average final weight, gm.	Days on KI	Average mitoses/ thyroid	Average parathyroid count: Mitoses/ 10,000 cells	Average parathyroid count: Cells/ unit field	Number of guinea pigs	Average final weight, gm.	Days on KI	Average mitoses/ thyroid	Average parathyroid count: Mitoses/ 10,000 cells	Average parathyroid count: Cells/ unit field	Number of guinea pigs	Average final weight, gm.	Days on KI	Average mitoses/ thyroid	Average parathyroid count: Mitoses/ 10,000 cells	Average parathyroid count: Cells/ unit field
Group I. Initial weight 105–115 gm.																	
3	118	5	113	1.0	208	2	116	5	230	1.3	225	2	125	5	430	2.0	200
2	124	10	232	2.2	210	2	126	10	480	1.1	179	2	140	10	650	2.2	180
3	137	15	633	3.1	207	2	145	15	670	2.5	160	2	185	15	820	3.3	158
2	148	20	310	2.0	200	2	170	20	550	1.9	210	2	220	20	560	1.4	145
2	203	30	120	2.1	195	2	235	30	265	0.8	240	2	245	30	190	0	150
2	210	40	40	0.9	230	2	245	40	120	0	230	2	280	40	150	0	180
		Average	273	1.9	208			Average	384	1.3	216			Average	467	1.5	169
Group II. Initial weight 180–200 gm.																	
6	209	4–7	176	2.7	189	6	180	4–7	285	2.4	210	4	229	4–7	460	3.3	190
4	224	10–12	248	2.5	195	10	212	10–12	449	4.3	204	8	251	10–12	812	4.1	195
4	225	15	885	4.2	192	5	225	15	963	6.7	180	6	271	15	1387	9.6	171
4	232	20	500	3.4	190	6	246	20	336	3.4	175	6	282	20	647	5.0	195
		Average	422	3.1	191			Average	483	4.1	194			Average	856	5.5	188
Group III. Initial weight 385–420 gm.																	
2		5	480	2.4	190												
2		10	1620	4.7	160												
4		15	2640	9.5	135												
4		30	1060	3.2	144												
2		40	400	1.9	210												
2		50	280	0.9	217												
		Average	1276	4.6	167												

and 200 gm. in weight, and between 4 and 6 weeks in age; *Group III* (24 guinea pigs) varied in weight between 390 and 420 gm. and were approximately 3.5 to 4 months of age. Three further subdivisions were made according to the diet given. Each of these three subdivisions received a basic diet consisting of lettuce, carrots and oats. Subdivision *A* received only the basic diet; subdivision *B* received in addition Purina rabbit chow containing wheat germ, soybean oil meal, cracked corn, molasses, 0.5 per cent calcium carbonate, and other salts which were iodine-free, while subdivision *C* received in addition to the basic diet the same Purina Chow, except that 0.5 per cent iodized salt replaced the iodine-free salt. (See table 1.)

In a second experiment, 123 guinea pigs were divided in a similar manner according to weight (age) and diet, and were fed their respective diets for a period of 10 days preceding the administration of KI. The latter substance was then administered either by feeding tablets or in solution through a tube introduced into the stomach, in doses of 0.01 or 0.05 gm., daily for periods varying between 5 and 50 days (see table 2).

The thyroid, parathyroid and adrenal glands were removed, immediately fixed and prepared for study by the paraffin section technique in the usual manner. Mitotic counts were made on these three glands according to methods described in previous publications (14, 15). Thyroid counts were recorded as the number of mitoses per total gland (both lobes), adrenal counts as the average number of mitoses per section, and parathyroid counts as the number of mitoses per 10,000 cells; in the latter instance, an additional figure representing the average number of cells per standard unit field was recorded in order to indicate changes in cell size.

EXPERIMENTAL RESULTS

Influence of very small amounts of iodine in the diet on mitotic activity in thyroid and parathyroid glands of normal male guinea pigs of various weights (ages). The influence of the iodine content of the diet on mitotic activity in the thyroid and parathyroid glands is shown in table 1. In *Groups I* and *II*, mitotic counts were made in guinea pigs in a manner similar to those used in the experiments in which guinea pigs of a corresponding age were fed with the various diets; these control counts serve as a baseline for the curves indicating the influence of the special diets. It can be seen that in *Groups I* and *II* the guinea pigs which received a diet not containing iodized salt show, on the average, a lower degree of mitotic activity of the thyroid gland than do those receiving the diet containing additional iodine. In *Group III*, in which only two diets were tested, no effect of the added iodine was observed. The reason for this becomes apparent when it is noted that in all three subdivisions there is a decrease in mitotic activity with increasing age, which occurs irrespective of diet. Apparently the factors which bring about this diminution in mitotic activity with increasing age are able to overcome the stimulating effects of iodine when a certain age has been reached. With this interpretation agrees the fact that in *Group II* the average increase in mitoses, resulting from the administration of the iodized-salt diet, is less marked than in *Group I*.

It may be noted further in table 1 that in subdivisions *A* and *B* of the youngest age group (*Group I*), which received diets to which iodine had not been added and which had therefore, a very low iodine content, there is a rapid fall in mitotic activity in the thyroid gland within the first 5 days, and this is maintained for somewhere between 20 and 30 days; at about 30 days there follows a sudden rise in mitotic activity, which subsequently again declines, without, however, reaching the same low level which it had at earlier periods. While the reason for this rapid rise is not definite, it may perhaps be a response of this gland to factors which become active

at the onset of sexual activity. On the other hand, in subdivision C of the lowest age group, there is a steady rise in mitotic activity through the 40 days during which these guinea pigs received the diet containing iodized salt. But even in this group the marked rise in mitotic activity developing between the 30th and 40th day may perhaps be attributed also to the onset of sexual activity. In *Group II*, the fall in mitotic activity of the thyroid gland in guinea pigs kept on non-iodized diets in subdivision *A* begins between the 10th and 15th day and continues until the 40th day, while in subdivision *B* there is a fall between the 25th and 40th days. The guinea pigs in *Group II*, subdivision C, which had received the iodized chow diet, show a steady rise in mitotic activity up to the 35th day, when the maximum is reached.

In general, the mitotic activity in the parathryoid gland in these experiments parallels that in the thyroid gland. Thus, in the parathyroid gland in *Groups I* and *II*, in the guinea pigs which received the non-iodized diet, the average number of mitoses is somewhat lower than in subdivision C which received iodized chow; likewise, the averages in age *Group II* are lower than those in the younger age *Group I*. The averages are lowest in the oldest age *Group III*, and here there was no increase in mitotic activity in subdivision C which received iodized chow. There is, furthermore, a correspondence between the average of cell size and the number of mitoses; an increased average number of mitoses is usually associated with an increased average cell size.

In the parathyroid as well as in the thyroid glands of the guinea pigs of the youngest age group (*Group I*) there is in subdivisions *A* and *B* the same rapid fall in mitotic activity within the first 5 days, due to a very low iodine intake and here, also it is maintained for about 20 to 30 days and is followed by a sudden rise in mitotic activity at about 30 days. As in the thyroid gland, mitotic activity subsequently declines, but not to the same level as at earlier periods. In subdivisions C of *Group I* in which the guinea pigs received iodized chow, there is a steady rise in mitotic activity, which again closely parallels that observed in the thyroid gland including the marked rise in proliferative activity between the 30th and 40th days, which may perhaps be attributed to the onset of sexual activity.

We may then conclude that in the age *Groups I* and *II*, the iodized chow causes an increase in mitoses in the thyroid and parathyroid glands, which is absolutely and also relatively greater in age *Group I* than in age *Group II*. In age *Group III* the mitotic activity is low in all subdivisions, in accordance with the greater age of these animals. In this latter group, the mitotic activity is not increased by substituting iodized chow for a non-iodized diet. In age *Group I* a maximum in mitotic activity is reached usually somewhere between the 25th and 40th days; at 40 days there occurs a fall from the maximum, which may perhaps be connected with the stage of sexual maturity reached at that time. There is also a decline in mitotic activity between the 1st and 5th days of observation in certain groups; however, the significance of this fall remains doubtful.

Effect on thyroid and parathyroid of the addition of large amounts of KI to the three basic diets of guinea pigs differing in age. These guinea pigs received either 0.01 or 0.05 gm. of KI daily for periods ranging from 5 to 50 days. This substance was administered either in the form of tablets orally, or as a solution by means of a tube inserted into the stomach. No significant difference was noted between the two doses used or between the two methods of administration, hence no distinction is made as to dose or method of administration in the discussion of the results (table 2).

In this table is shown the influence on the mitotic response in thyroid and parathyroid glands of the various amounts of KI given to guinea pigs kept on the various diets. Mitotic activity in the thyroid glands of guinea pigs in subdivision *A* of

Groups I and *II* was somewhat less than in those in subdivision *B*, while mitotic activity in the latter was, in turn, somewhat less than that in guinea pigs of subdivision C. Here the average mitotic counts were highest. There was a summation of the effects of iodized chow and of the additional KI. However, in age *Group II*, subdivision C, the effects were greater than those of the mere summation. This seems to be due to the influence of age (weight) on the response to iodine; the old guinea pigs responded with a greater increase in mitoses to the addition of KI. This comes out most markedly in the oldest age group, in which, even in subdivision A, the averages reach the highest level, namely, 1276 mitoses. In general, it can be seen that the maximum degree of mitotic activity in the thyroid of all groups is reached after about 15 days of KI administration. This corresponds to the former experiments on guinea pigs in which it has been found that the largest number of mitoses were observed after KI had been given orally for a period of 15 days.

The difference in the degree of mitotic activity in the various age groups should especially be noted. The average maximum mitotic response in *Group I* in the animals receiving iodized chow (subdivision C) was 820 mitoses per thyroid gland and 3.3 per 10,000 cells in the parathyroid, whereas, in *Group II* it was 1387 in the thyroid and 9.6 per 10,000 cells in the parathyroid. In *Group III*, in which only one non-iodized diet was given, the maximum response was greater than in the two groups of younger guinea pigs (2640 mitoses per thyroid gland on the average, and 9.5 mitoses per 10,000 cells in the parathyroid gland). There was, then, in response to the administration of KI, an increase in mitotic activity in both the thyroid and parathyroid glands with increasing weight (age) in these experiments. There is a parallelism between the mitotic activity in thyroid and parathyroid in that also in the parathyroid the maximum number of mitoses was found after 15 days and that in *Group I*, the youngest age group, the number of mitoses reached the lowest average level. Furthermore, in the second age group the number of mitoses increased in the direction from the other two diets to the iodized chow, and among the animals in subdivision A the average counts increased with increasing age. There is, then, in the most essential respects, a parallelism between the effects of iodine on the mitotic activity of thyroid and parathyroid.

The relationship between the daily dose of KI and the intensity of the mitotic response in the thyroid and parathyroid glands. In the various experiments carried out by Loeb and his associates, Gray (6), Rabinovitch (7), McCordock (13), and Margolin (12), the results which follow the administration of KI to guinea pigs during the initial period were concordant as far as the increase in mitoses in the thyroid gland is concerned. There is also agreement as to the time when the maximum response of the thyroid is observed, namely, after about 15 days of oral administration of this substance. On the other hand, there are great differences as to the degree with which the mitotic activity was increased in different experiments. Thus, Rabinovitch (7) reported maximum figures for mitotic counts as high as 4000 to 8000 mitoses per thyroid gland when the optimal doses of KI, namely 0.1 gm. daily, was given. The figures found by Gray and Loeb (6), as well as by McCordock (13), were lower; Margolin (12) subsequently reported that guinea pigs fed as little as 0.0005 gm. of KI daily for 15 days showed an average count of 1188 mitoses, a degree of response similar to that observed by other investigators with 0.01 and 0.05 gm. of KI. Our own counts (table 1) indicate that the weight of the guinea pigs used is probably one of the variable factors which is responsible for variations in the mitotic counts. It was also of interest to determine the influence of the daily dose of KI on the mitotic activity of the thyroid gland, in view also of the finding of Rabinovitch (7) that within a certain range the increase in the dose of KI increased with the number of

mitoses. In an additional experiment in which 24 guinea pigs were used, the initial weight of the guinea pigs was approximately the same as that of *Group II* corresponding to an age of 4 to 6 weeks. These animals were fed the diet given to subdivision A, not containing chow. We added also some of the counts of 16 other guinea pigs shown in table 1. The guinea pigs received KI daily for periods of 15 and 20 or 25 days. The results in table 3 indicate that the maximum is recorded usually after 15 days of administration of this substance and that even a daily dose as low as 3.5 to 7.0×10^{-6} gm. of KI still has some effect. There was no essential difference between

TABLE 3. RELATIONSHIP OF THE DAILY AMOUNT OF KI ADMINISTERED TO THE DEGREE OF MITOTIC RESPONSE IN THYROID AND PARATHYROID GLANDS

Number of Guinea Pigs	Daily Amount of KI Fed, gm.	Number of Days Fed KI	Thyroid Count, Mitoses/ Gland		Parathyroid Count			
					Mitoses/10,000 cells		Cells/unit field	
			Number	Average	Number	Average	Number	Average
Initial weight 180–190 gm.; final weight 220–285 gm.								
2	10^{-1}	15	580	710	3.4	5.2	193	184
2	10^{-1}	20	840		6.9		174	
2	5×10^{-2}	15	647	635	3.8	3.4	201	202
2	5×10^{-2}	20	600		2.9		203	
2	10^{-2}	15	907	752	4.5	3.8	190	194
2	10^{-2}	20	520		3.1		198	
3	5×10^{-3}	15	913		5.8		158	
3	10^{-3}	15	407	324	2.5	2.3	195	189
2	10^{-3}	20	200		2.1		181	
2	10^{-4}	15	500	370	4.1	3.2	150	168
2	10^{-4}	20	240		2.3		185	
6	Chow with KI[1]	15	210	232	2.6	2.5	176	176
6	Chow with KI[1]	25	254		2.4		175	
2	No chow Controls	15	130	115	1.1	1.3	195	190
2	No chow Controls	25	110		1.4		185	

[1] This chow furnishes a daily intake of 3.5 to 7.0×10^{-6} gm. of KI per guinea pig.

responses to doses of 10^{-1} and 5×10^{-3} gm.; however, if still smaller amounts of KI are given, the effectiveness decreases, and it reaches the lowest point if the iodized chow is used. These results apply in the case of guinea pigs in the weight and age range chosen in these experiments. It is possible that if heavier and older animals are used, such as those used by Rabinovitch, the results may not be exactly the same.

If we compare the mitotic activity in the parathyroid gland with that in the thyroid, we find a very close parallelism. However, the correspondence is not in every respect complete and this may be taken as an indication that there may be an additional factor determining the intensity of mitotic proliferation which is not identical in thyroid and parathyroid. As to the size of the parathyroid cells in these experiments, the variations were relatively slight and are probably of no significance.

The response of the adrenal cortex to KI administration. One hundred and five guinea pigs received KI tablets by mouth in doses of 0.01 or 0.05 gm. for periods ranging from 4 to 20 days (table 4). The total average number of mitoses per section

of adrenal gland in the KI-fed guinea pigs was 3.1 as compared with 2.7 in 72 control guinea pigs. This difference is not very great and the number of variations in the different groups is considerable and overlapping. However, a statistical analysis of these results, as indicated in the last column of table 4, shows that there is a significant increase in mitotic activity between the 8th and 15th day of KI administration. A further statistical comparison of the results in the group receiving KI for 8 to 10 days with those in the group receiving this substance for 12 to 15 days showed a figure of 1.7, which is not significant. It may therefore be concluded from these results that KI has some effect on mitotic activity in the adrenal cortex of the guinea

TABLE 4. MITOTIC COUNTS IN THE ADRENAL CORTEX OF CONTROL AND KI-FED GUINEA PIGS

Number of Guinea Pigs	Number of Days Fed KI	Average Number of Mitoses per Section		Range of Variation in Average Number of Mitoses per Section	Factor of Statistical Significance as Compared with Controls[1]
Initial weight 180–200 gm.; final weight 185–365 gm.					
25	4–7	2.1		0– 6.8	1.0
22	8–10	4.3	Av.	0.2–17.2	3.5
36	12–15	3.3	3.1	0–11.5	4.3
22	18–20	3.0		0– 8.7	1.1
72	Controls	2.7		0–10	

[1] The factor of statistical significance was calculated according to the relationship $A_1 - A_2/SE_{A_1} - SE_{A_2}$, in which A represents the average mitotic count for a given group and SE the standard error for the corresponding group. If this factor is less than 2, there is no significant difference between the two groups compared, while if it is greater than 3, it is almost certainly significant. A factor between 2 and 3 indicates a probability greater than merely a chance phenomenon.

pig, and that this increase in proliferative activity is manifested between the 8th and 15th days of KI administration.

DISCUSSION

As stated previously, Loeb and his associates (6–13) have observed that the oral administration of KI results in an increase in mitotic activity in the thyroid gland in guinea pigs, which reaches a maximum between the 15th and 20th days. There are, however, considerable variations in the findings of different investigators as to the degree of this maximum response. Rabinovitch (7) noted that the maximum count was sometimes as high as 8000, and quite commonly reached figures of 4000 and 5000 mitoses per thyroid gland; in certain of his experiments the guinea pig weighed between 350 and 450 gm. Similarly, Gray and Rabinovitch (8) subsequently found an average of 3442 mitoses per thyroid gland in two guinea pigs fed KI for 20 days. On the other hand, Gray and Loeb (6) obtained an average maximum figure of only 710 mitoses per thyroid gland in guinea pigs fed KI for 16 to 23 days, and McCordock (12) observed an average count of 1208 mitoses per thyroid gland in guinea pigs which had received KI for 15 to 20 days. In the latter experiments the weight or age of the guinea pigs used was not given.

In the present experiments it has been shown that weight (age) is an important factor in determining the degree of mitotic response to KI administration. The intensity of this response apparently depends to a great extent upon two antagonistic influences, namely, the stimulating action of KI, and the factors concerned in ageing, which tend to lower mitotic activity. In the first group of experiments (table 1), in which certain guinea pigs were fed diets very low in iodine content, the ageing fac-

tors were predominent and there was a gradual diminution in mitotic activity in the thyroid gland with increasing age. Yet, in guinea pigs receiving a diet of iodized chow the small amount of iodine contained in the diet was sufficient to produce an increase in mitotic activity; this applied so far as the two younger age classes were concerned. But in guinea pigs weighing about 400 gm. such a response was no longer obtained; here the ageing factors predominated over the stimulating effects of the small amounts of KI present in the chow diet.

It seems, however, that the depressing effects of ageing can be overcome by increasing the amount of iodine administered, and, indeed, when this is done the response in older animals becomes even greater than in younger ones. This effect of the age of guinea pigs on the degree of mitotic proliferation of the thyroid glands could account in some degree for the variations in the results of the investigators mentioned above, but it does not completely explain the differences, since the maximum mitotic activity in the oldest age group in the present experiments still does not reach the high degree of mitotic response to KI reported by Rabinovitch (7, 9). It may be that still older animals would have yielded results comparable to those observed by the latter investigator. It will be of interest in future experiments to determine the proliferative reaction of the thyroid gland to KI in still older groups of guinea pigs than those we have used so far.

It is also of great interest that the very small amount of iodine available in the chow containing iodized salt was sufficient to result in a measurable increase in mitotic activity in the thyroid gland. Margolin (12) has reported that doses of KI as small as .0001 gm. per day may have a slight stimulating effect on mitosis. We have also shown that amounts as small as 10^{-4} gm. of KI administered daily by mouth are effective, and that even still smaller quantities of KI, such as those present in the diet of iodized chow, may exert a distinct effect. The preparation of chow used in these experiments contained 88 micrograms of KI per 100 gm. of chow. The average daily consumption of chow in addition to the other elements in the diet was approximately 4 to 8 gm. The average daily KI intake from this source was therefore between 3.5 and 7.0 μg. While it cannot be excluded that small amounts of iodine may also have been present in the lettuce, carrots and oats used in these diets, still, this very small increase in the daily total iodine intake provided by the chow was sufficient to result in a definite stimulation of the mitotic activity of the thyroid gland. In the present experiments it has been shown that within a rather wide range, larger doses produce a more marked effect than do smaller doses, while the latter also lead to an increase in mitotic activity when compared with control animals receiving a diet which does not contain iodized salt. In this case, also, additional experiments are necessary to determine the minimal amount of KI which will result in an increased mitotic activity in the thyroid gland.

It has, furthermore, been observed in these experiments that guinea pigs maintained on the diet containing iodized chow respond with greater intensity to the subsequent administration of larger doses of KI than do guinea pigs maintained on the diets not containing iodized salt. This may be due to a cumulative effect of the iodine in the diet and the iodine subsequently administered in the form of potassium iodide, or it may be that the thyroid glands of guinea pigs maintained on diets very low in iodine become so depressed functionally that they do not respond with as great an intensity to subsequent stimulation with iodine as glands which have been previously supplied with very small amounts of iodine.

In a general way, the mitotic response of the parathyroid to various daily doses of KI was parallel to that of the thyroid gland, although there were some exceptions. The parallelism in the response of the thyroid and parathyroid glands to various

hormones and hormone-like substances, which has recently been reported by Loeb and the author (14) and which is confirmed in the present experiments as far as KI is concerned, assumes added importance in the light of certain clinical and experimental observations. Beaumont, Dodds and Robertson (16) have reported a number of clinical cases in which there was an alteration of calcium and phosphorus metabolism in thyrotoxicosis, and Mansbacher (17) has recently published a report of cases of osteoporosis in hyperthyroidism. Michaud (18), Hansman and Wilson (19), Puppel and Curtis (29), Cope and Donaldson (21) and others have demonstrated that increased activity of the thyroid gland is associated with an increase in the excretion of calcium and phosphorus. Aub, Bauer, Heath, and Ropes (22), Collip (23), Langdon-Brown (24), and Cope and Donaldson (21) are of the opinion that the increased excretion of calcium in thyrotoxicosis depends on a direct stimulating catabolic effect of the thyroid secretion on the calcium deposits in the bones. On the other hand, according to Beaumont, Dodds and Robertson (16) 'some other cause than the direct action of secretion must be sought to account for the decalcification and excessive calcium and phosphorus loss from the body in thyrotoxicosis.' The experiments of Loeb and the author (14) as well as the present experiments indicate that these alterations in calcium and phosphorus metabolism may perhaps be due to the concomitant increase in parathyroid activity, which occurs, at least in the guinea pig, when the thyroid gland is stimulated to increased proliferative activity. However, further investigations are necessary before any definite conclusions can be drawn as to the mechanism of the altered calcium and phosphorous metabolism which is associated with hyperthyroidism.

Our purpose in including experiments concerning the effect of KI on mitotic activity in the adrenal cortex was to determine whether or not the mitotic activity activity of the adrenal cortex parallels that of the thyroid gland in a manner similar to that of the parathyroid. That the proliferative activity of the thyroid gland may have some influence on the activity of the adrenal cortex has been indicated by the experiments of McQueen-Williams (25), Emery and Winter (26), Rosen and Marine (27) and others. These workers have reported that there was either an absence or a decreased response of the adrenal cortex to the administration of pituitary substance following thyroidectomy. The present investigations indicate that the increase in proliferative activity of the thyroid gland which results from KI administration is in all probability accompanied by a slight increase in mitotic activity in the adrenal cortex. However, subsequently we shall discuss in greater detail the relationship of the thyroid gland to the adrenal cortex.

SUMMARY

1. The addition of very small amounts of KI in the form of iodized chow to the diet of male guinea pigs between the ages of 2 and 6 weeks results in an increase in proliferative activity in the thyroid and parathyroid glands. Older guinea pigs (3 to 4 months old) do not respond to such small doses of KI.

2. Guinea pigs maintained on diets to which no iodine has been added do not respond as intensely to subsequent administration of larger quantities of KI (0.01 and 0.05 gm.) as do guinea pigs maintained on a diet containing iodized chow.

3. When the larger doses of KI (0.01 and 0.05 gm.) are administered daily, a typical maximal response is observed in both thyroid and parathyroid glands at about the 15th day, which is then followed by retrogression. This response increases with increasing age, within the age limits tested in these experiments.

4. No strict parallelism was observed between the amount of KI administered daily and the degree of mitotic activity observed in the thyroid and parathyroid

glands. However, within a rather wide range, larger doses of KI produce more marked activity in the thyroid and parathyroid glands than do smaller doses.

5. These experiments confirm the previous observation that a parallelism exists between the effect of KI on the thyroid and parathyroid glands; in addition, it is shown that potassium iodide causes in all probability a slight increase in the mitotic activity of the adrenal cortex from the 8th to the 15th day of the administration of this substance.

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RELATION OF THYROID TO GROWTH

I. EFFECTS OF CRYSTALLINE THYROXIN UPON RATE OF GROWTH, FOOD INTAKE AND BODY COMPOSITION OF FEMALE ALBINO MICE[1,2]

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IT HAS BEEN KNOWN for many years that small amounts of thyroid material exert an anabolic effect when administered to hypothyroid animals. The literature is not in agreement, however, upon the effects when thyroid material is given to normal animals. Using body weight as an indication of activity, most investigators have concluded that thyroid material has a 'catabolic' effect, *i.e.*, it will cause a suppression of growth or an actual loss of body tissue when fed to intact laboratory animals. The literature on this subject is extensive. For reviews see references 1,2 and 3.

Nevertheless, there have been a few notable exceptions to this general agreement. Moussu (4) and Dott (5) reported that feeding small amounts of thyroid tissue to immature dogs always resulted in acceleration of the growth rate. Dott (5) concluded that the early rapid growth was followed by premature closure of the epiphysis of the long bones resulting in a small mature stature. Moussu, however, observed that the maximum size attained by his animals was normal for the breed. Both investigators used bone measurements as an index of growth.

Similar results have been obtained with rats by a few investigators. Schafer (6), in 1912 reported that the rate of growth and feed intake of female rats were markedly increased by feeding small amounts of thyroid. Males were unaffected. Hoskins (2) confirmed these results but the differences observed between treated and untreated animals were not as great as those reported by Schafer (6). More variable results were obtained by Herring (7). Some of his animals grew rapidly while the growth rate of others was apparently depressed.

Increased rates of growth of mice was reported by Robertson (8). From an extensive series of trials he concluded that growth of immature male and female mice was accelerated by feeding an equivalent of 1.9 mg. of fresh thyroid daily and that longevity was reduced by approximately 17 per cent.

Certain clinical reports during recent years indicate that the results mentioned above are not attributable to isolated responses having no general application. Evidence has accumulated that mild hyperthyroidism (induced or spontaneous) results in increased nitrogen retention and growth of children (9–12). In addition, it has been demonstrated in this laboratory and by others that proper amounts of crystalline thyroxin (13–16), dried thyroid (14, 17), or thyroprotein (18) will induce an increase in the rate of milk secretion of cows or goats. Consideration of these reports, along with certain pituitary relationships (discussed in a later section of this paper), gave

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rise to the question of whether increased growth of immature animals could be obtained consistently by giving the proper amount of thyroid material. This communication presents our observations upon this subject, including the efficiency of growth and changes in body composition associated with thyroxin treatment.

PROCEDURE

Virgin female mice of unknown ancestry and weighing from 10 to 15 gm. were purchased from a commercial breeding laboratory as needed. The animals were housed in basement laboratories where the temperature usually remained between 78° and 85° F. Two or three days were allowed for recovery from shipment after which time the animals were separated into groups of 10 to 12 each, given individual numbers, weighed, and placed on experiment.

The control and treated animals were either caged separately and the cages kept side by side in the laboratory or else one-half of the animals in each cage were treated and the remainder were left as controls. The treated animals received subcutaneous injections of 0.1 cc. of a suspension of crystalline thyroxin at its isoelectric point. The controls were uninjected. All animals were weighed one or more times weekly.

Experiment 1 was started in August, 1941. Four groups of mice received daily injections of 0.007, 0.009, 0.02, 0.04 mg. of thyroxin, respectively. One group was untreated and served as controls. The animals were fed a mixed ground feed and a commercial dog feed in the form of pellets *ad libitum*.

Experiment 2 began in September, 1941, and consisted of one control group and one experimental group. The treated animals received 0.03 mg. of thyroxin on alternate days. The food was the same as that used in *experiment 1*.

Experiment 3 was started in November, 1941, and was a duplicate of *experiment 2* with the exception that the thyroxin dosage was increased on the fifteenth day of treatment from 0.03 to 0.06 mg. of thyroxin on alternate days.

Experiment 4 was begun in January, 1942. The thyroxin injection was increased from 0.03 to 0.06 mg. of thyroxin on alternate days on the 10th day of treatment. Food intake was determined for each group of 10 animals in this trial. Ground food only was allowed and this was fed in deep pint fruit jars with lids that barely permitted the mice to pass to and from the food containers. The amount of food wasted was small.

Experiment 5 was started in February, 1942, and consisted of one control and one experimental group. Thyroxin treatment was the same as in *experiment 4*. The animals were fed *ad libitum* both ground food and pellets as in *experiments 1, 2,* and 3.

The number of animals used is shown in table 1.

Seven groups of mice were killed for determination of body composition. Five groups were taken from *experiment 4*, one group being killed at the beginning of the experiment, and a group each of treated and control animals being taken on the 27th and 43rd day of treatment, respectively. A group each of injected and of non-treated animals was removed from *experiment 3*, 93 days after treatment began.

The animals were killed with natural gas, the gut removed and stripped of its contents and returned to the carcass. The combined weight of the empty carcass and digestive tract is referred to as the carcass weight. The bodies of the animals were then pooled by groups, ground in a meat chopper, dried to constant weight at 60° C., reground in a mill and analyzed for nitrogen, fat, and ash. Nitrogen was determined by the macro-Kjeldahl method, fat by extraction with petroleum ether in a Soxhlet apparatus, and ash by ignition in a muffle furnace. Protein was calculated by using the nitrogen factor 6.25. Energy values were estimated using the factors of Carpenter

(19) for protein, fat, and carbohydrate. Any dry tissue not accounted for by protein, fat and ash was arbitrarily assumed to be carbohydrate.

OBSERVATIONS

Growth. A summary of the results obtained on growth is presented in figure 1 and table 1. The observations reported include only those made during the first 5 weeks of experimental treatment since this investigation was confined primarily to the period of rapid growth and development.

TABLE 1. BODY WEIGHT GAINS OF THYROXIN-INJECTED AND CONTROL MICE DURING 5-WEEK PERIODS

Experiment	Treatment, Daily	Number Animals	Initial Weight	Weight at End of 5 Weeks	Gain
			gm.	gm.	gm.
1	Controls	9	15.9±1.03[1]	20.9±2.14	5.0±1.95
	0.04 mg. Thyroxin	11	16.5±1.24	23.6±2.71	7.1±2.12
	0.02 mg. Thyroxin	11	17.4±0.75	24.9±1.58	7.5±1.49
	0.009 mg. Thyroxin	11	17.3±1.27	24.6±1.10	7.3±1.22
	0.007 mg. Thyroxin	12	16.7±1.18	22.4±1.87	5.7±1.37
2	Control	29	15.7±1.36	21.4±1.89	5.7±1.92
	0.015 mg. Thyroxin[2]	28	14.9±1.30	21.9±1.68	7.0±1.56
3	Controls	13	15.0±1.18	20.3±2.41	5.3±1.97
	0.015–0.030 mg. Thyroxin	15	14.5±1.54	21.7±3.12	7.2±2.30
4	Controls	10	14.2±1.07	17.8±2.13	3.6±1.66
	0.015–0.03 mg. Thyroxin	8	13.9±0.91	18.4±2.98	4.5±2.89
5	Controls	9	14.1±1.34	19.9±1.81	5.8±1.34
	0.013–0.030 mg. Thyroxin[2]	9	13.7±0.70	20.9±2.39	7.2±2.19
Total	Controls	70	15.1±1.40	20.4±2.29	5.3±1.94
	Treated	105	15.6±1.74	22.4±2.70	6.8±1.97

[1] All such figures represent standard deviations.
[2] Actually, twice these amounts were given on alternate days. See figure 1.

It will be noted from figure 1 that the thyroxin-injected animals consistently and repeatedly grew at a more rapid rate than did the controls. Under the terms of these experiments the treated animals gained an average of 28 per cent more weight during 5 weeks than the controls. Statistical treatment showed this difference to be highly significant. Continued treatment and observation of the animals in *experiments 2* and 3 showed that this difference remained stable for a few weeks after which time the difference became less as the weight of the controls approached that of the treated animals. Thus our observations have confirmed those of Moussu (4) and Robertson (8) that thyroid-treated animals reach their maximum weight at an earlier date, but do not exceed that finally attained by the controls.

The cause of the temporary loss in weight of the experimental animals in *experiment 1* is not known. No changes in treatment, temperature or other environmental conditions were observed. It will be noted that the loss in weight was proportional to the thyroxin injected. Other preliminary work has indicated that mice receiving more than 0.03 mg. of thyroxin daily are sensitive to environmental changes. In this connection it is emphasized that the results reported in this communication were obtained under certain controlled conditions and that a different environment might have altered the results obtained.

The rate of growth of both control and experimental animals in *experiment 4* was below that of mice in the other trials. This reduction is attributed to decreased food intake due to conditions imposed to measure food consumption.

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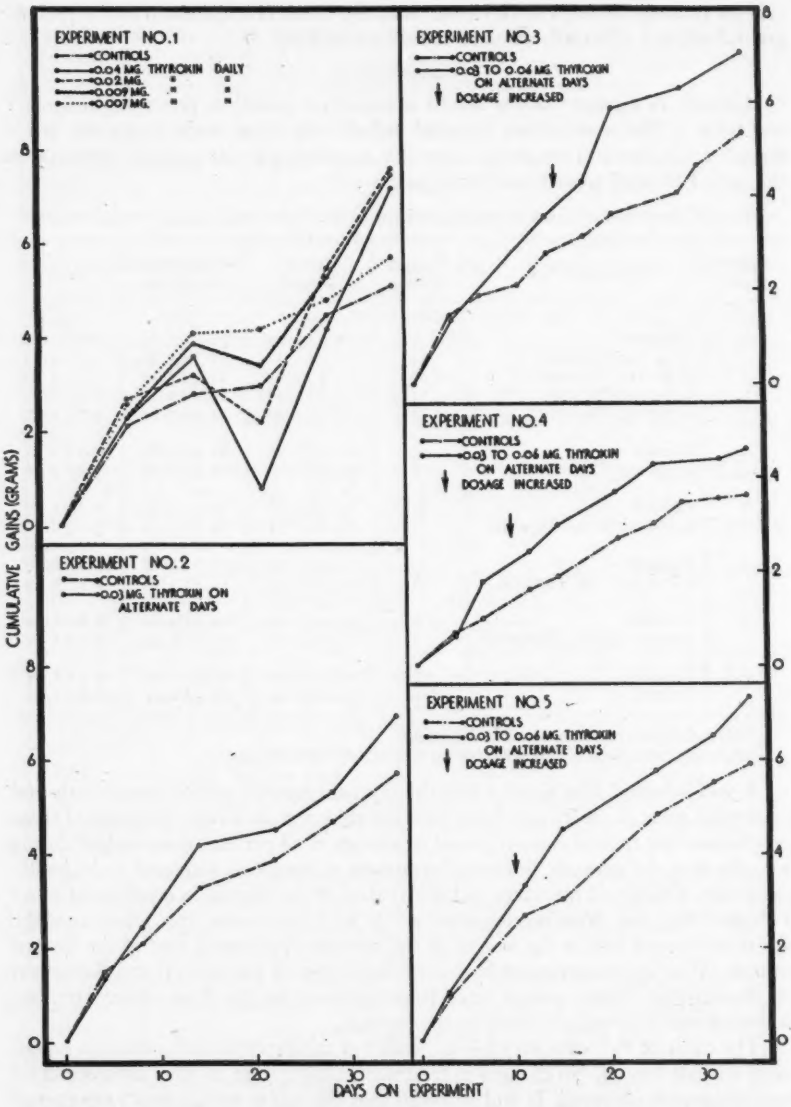

Fig. 1. Average growth curves of thyroxin-treated and control mice. Each circle represents the average for the group. The dosages given represent the amount injected per mouse. For number of animals involved see table 1.

Food intake. Food intake was determined for 4 cages of 10 mice each in *experiment* 4 for 27 days. At this time two groups were removed for analysis and two groups left for the remainder of a 43-day period. Under the condition of this trial, the control mice consumed an average of 3.64 gm. of food per day per mouse while thyroxin-treated mice ate an average of 4.55 gm., an increase of approximately 25 per cent.

TABLE 2. COMPOSITION OF TISSUES OF CONTROL AND THYROXIN-INJECTED MICE

	13–16 gm.	27 Day[1]		43 Day[1]		93 Day[1]	
	Controls	Controls	Treated	Controls	Treated	Controls	Treated
Weight of Carcass, gm.	12.7	15.7	16.9	17.2	18.0	21.0	22.0
Dry Matter in Carcass, %	32.4	36.3	34.3	37.5	34.3	35.7	34.4
Composition of Dry Carcass							
Nitrogen, per cent	9.07	8.65	9.54	7.64	8.56	8.26	8.67
Protein, per cent	56.7	54.0	59.6	47.8	53.5	51.6	54.2
Fat, per cent	26.3	30.4	26.1	31.5	34.1	27.4	27.3
Ash, per cent	10.6	10.6	10.3	9.0	10.0	9.9	10.6
Energy, Cal. per 100 gm.	597.4	614.5	601.5	618.4	635.8	598.8	598.8
Composition of Fresh Carcass							
Nitrogen, per cent	2.93	3.14	3.27	2.86	2.95	2.95	2.96
Protein, per cent	18.3	19.6	20.5	17.9	18.4	18.4	18.7
Fat, per cent	8.5	11.0	9.0	11.8	11.7	9.8	9.4
Ash, per cent	3.4	3.9	3.5	3.4	3.4	3.4	3.7
Energy, Cal. per 100 gm.	192.6	224.0	206.4	231.7	218.1	213.5	206.2
Amount per Mouse							
Dry matter, gm.	4.09	5.70	5.80	6.44	6.17	7.49	7.58
Nitrogen, gm.	0.37	0.49	0.55	0.49	0.53	0.62	0.65
Protein, gm.	2.32	3.06	3.46	3.08	3.31	3.87	4.10
Fat, gm.	1.08	1.73	1.51	2.03	2.10	2.05	2.07
Ash, gm.	0.43	0.61	0.60	0.58	0.62	0.71	0.81
Energy, Calories	24.46	35.16	34.87	39.85	39.26	44.8	45.4

[1] Number of days on experiment before autopsy.

Composition of body tissue. The composition of the carcasses of the different groups of mice is shown in table 2. The fresh carcasses of the thyroxin-treated mice contained a greater percentage of nitrogen and water, and a lesser percentage of fat than did their controls. Thus the treated mice more nearly maintained the composition of younger mice.

The total amounts of the constituent elements in the different groups are shown in the lower section of table 2. Comparison of the different pairs in a group will give an estimate of the relative amounts stored by the treated and non-treated mice, since the beginning weights were similar. More detailed calculations on this point are shown in table 3.

Inspection of tables 2 and 3 show that the thyroxin-treated animals stored considerably more nitrogen than did the controls. This is in agreement with the results of Johnston (11) with children, and it is of interest that Terroine and Babad (20) have reported marked retention of nitrogen by rats under the influence of thyroxin, even when the amounts given depressed body weight.

In contrast to the effects of thyroxin on nitrogen retention, fat was stored in greater amounts by the control animals. The energy retained by treated and non-treated animals was almost identical in the 3 group-pairs. Thus the increased growth of the treated animals was due to a change in the form of nutrients stored rather than to a general increase in all body tissues.

The amount of skeletal material, as indicated by ash analysis, was not markedly different in treated and non-treated animals during shorter periods of treatment. After longer periods of treatment, however, the ash content of the treated animals was greater than that of controls.

Efficiency of food utilization. Sufficient data are not available to make detailed cal-

TABLE 3. NUTRIENTS STORED BY THYROXIN-TREATED AND INJECTED MICE. VALUES REPRESENT AVERAGE PER MOUSE

	27 Day[1]		43 Day[1]	
	Controls	Treated	Controls	Treated
Food Intake, gm.	98.3	115.4	154.8	202.1
Body Weight, gm.				
a) At end	16.8	18.4	18.4	19.3
b) At beginning[2]	13.6	14.3	14.2	13.9
c) Gained	3.2	4.1	4.2	5.4
Ratio: $\frac{\text{body weight gained}}{\text{100 gm. food intake}}$	3.3	3.6	2.7	2.7
Protein, gm.				
a) At end	3.06	3.46	3.08	3.31
b) At beginning[2]	2.32	2.42	2.40	2.35
c) Gained	0.74	1.04	0.68	0.96
Ratio: $\frac{\text{protein gained}}{\text{100 gm. food intake}}$	0.75	0.90	0.44	0.48
Fat, gm.				
a) At end	1.73	1.51	2.03	2.10
b) At beginning[2]	1.08	1.27	1.12	1.10
c) Gained	0.65	0.24	0.91	1.00
Ratio: $\frac{\text{fat gained}}{\text{100 gm. food intake}}$	0.66	0.21	0.59	0.49
Energy, Calories				
a) At end	35.16	34.87	39.85	39.26
b) At beginning[2]	24.46	25.53	25.35	24.82
c) Gained	10.70	9.34	14.50	14.44
Ratio: $\frac{\text{energy gained}}{\text{100 gm. food intake}}$	10.89	8.10	9.37	7.14

[1] Number of days on experiment before killing.
[2] Estimated, using the weight of animals involved and the analysis of 13–16 gm. controls shown in table 2.

culations concerning the efficiency of utilization of the food nutrients. The amount of body weight increment, protein stored, and energy retained per 100 gm. of food intake was computed for 2 groups of thyroxin-treated mice and compared to that of controls. These computations are summarized in table 3.

The thyroxin-treated animals stored more protein and gained more body weight per unit of food intake than did the controls during the first 27 days of treatment. On the other hand, the controls stored more fat and more energy per unit of food intake than the treated animals. At the end of 43 days of treatment the amount of body weight increment and protein stored per unit of feed intake was similar in treated and non-treated animals. This fact emphasizes a practical implication, namely that thyroxin treatment will increase the apparent efficiency of body weight and nitrogen increments only during the time that the rate of growth is more rapid than in non-treated animals. From the standpoint of efficiency then, treatment should be stopped before the rate of gain decreases to that of normal controls. If thyroxin treatment is extended beyond this time, efficiency of food utilization by the thyroxin-treated animals will decrease due to the fact that they continue to eat more food than the non-treated.

It is recognized that the above gross comparisons are subject to valid criticism. First, balance trials were not conducted and consequently it cannot be determined whether the increased nitrogen storage was due to more complete absorption or to decreased excretion. Also, the apparent gross efficiency may be somewhat unfair in view of the fact that the treated mice were heavier and presumably had a greater maintenance cost. Nevertheless, the ratios calculated have a practical application Beginning with animals of the same weight, thyroxin-treated mice stored more nitrogen and gained more weight for each unit of food consumed than did non-treated animals. From an agricultural viewpoint, this is an interesting observation that may be of economic importance if farm animals respond in a like manner.

DISCUSSION

Using crystalline thyroxin we have obtained evidence that small amounts of thyroid material will increase the growth of normal animals. Furthermore, there is considerable indirect evidence that mild hyperthyroidism is conducive to rapid growth: *a*). The thyrotropic potency of the anterior pituitary is high during the period of rapid growth of rats (21), rabbits (22), and swine (23). *b*). In rats, a species wherein the male grows more rapidly than the female, the thyrotropic potency of the anterior pituitary is higher in the male than in the female (21). On the other hand, the thyrotropic potency and the rate of growth of rabbits are similar in the two sexes (22). *c*) The pituitaries of slow-growing strains of swine have been found to be lower in thyrotropic potency than those of faster growing animals (23). *d*) Hertz and Galli-Mainini (12) have presented evidence that hyperthyroid girls grow more rapidly than normal children.

The evidence cited is not an indication that all animals will respond to mild thyroid treatment with accelerated growth. Assuming that most hormonal factors normally have a margin of safety, one could conceive that mild thyroid treatment would ordinarily accelerate the rate of growth since it is beneficial in hypothyroidism. Some animals may be so genetically constituted, however, that they normally exist near the upper limit of thyroid tolerance. It was noted in our experiments that there were non-treated animals that made as large gains in weight as any of the treated animals but large gains were more prevalent among the thyroxin-treated animals. The fact that Herring (7), Hoskins (25), and Peiser (26) have noted variations in the response of different litters of rats to thyroid treatment suggests that different strains of animals within a species may differ in their inherited thyroid function or tolerance. In addition, environmental conditions such as temperature and humidity may influence the response to thyroid treatment.

If farm meat-producing animals should respond to thyroid treatment in a manner similar to mice, the use of such materials as thyroid-active iodo-proteins may find practical application in the livestock industry to induce rapid rate of growth and accumulation of protein material. Andrews and Bullard (27) have reported rapid fattening and gain in weight following partial thyroidectomy of steers. These reports suggest the possibility that a similar response would occur following withdrawal of thyroid treatment due to the suppression of the animal's thyroid gland by the thyroid medication (28).

SUMMARY

Virgin female mice weighing from 13 to 16 gm. were given subcutaneous injections of crystalline thyroxin in dosages ranging from 0.015 to 0.04 mg. daily. The thy-

roxin-treated animals repeatedly gained an average of 28 per cent more weight during a period of 5 weeks than did the controls. The maximum difference between the body weights of control and treated mice was attained in about 5 weeks after which time the difference gradually became less.

The daily food intake of the thyroxin-treated animals was approximately 25 per cent above that of the controls.

The carcasses of the treated animals had a higher percentage of water and nitrogen and a lower percentage of fat than those of the controls.

Thyroxin-treated mice stored more nitrogen and gained more in body weight per unit of food intake than did controls for the first few weeks of treatment. After the rapid gains of the treated animals had subsided, the controls were more efficient in this respect than treated animals. Non-treated animals stored more fat and more energy per unit of food intake than did the thyroxin-injected animals.

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CARBOHYDRATE METABOLISM

I. EFFECT OF B VITAMINS ON LIVER GLYCOGEN OF THYROID-FED RATS[1,2]

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RECENT STUDIES emphasizing the interrelationships between thyroid function and the metabolism of the B vitamins have shown *a*), that thyroid feeding decreases the amount of vitamin B_1 in rat tissues (1, 2), and *b*), that the food intake and weight maintenance of thyroid-fed rats depend on an adequate intake of the B vitamins (3), particularly vitamins B_1, B_6, and pantothenic acid (4). It has been repeatedly demonstrated that thyroid feeding will decrease the amount of glycogen in the liver (5, 6, 7, 8), and Abelin (9) and Abelin, Knochel, and Spichtin (10) reported that a yeast extract would prevent the fall in liver glycogen of rats that had been fed thyroid gland for 5 to 6 days. This observation was confirmed by Drill (11), who studied the liver glycogen of rats injected with small amounts of thyroxin. However, the above studies on thyroid-B vitamin interrelationships have demonstrated the need for additional work on the relationship of the liver glycogen of hyperthyroid rats to the B vitamins, particularly using larger doses of thyroid than previously employed and continuing the thyroid feeding for a longer period of time.

METHODS

Only female rats, averaging approximately 200 gm. in weight, were used, since it has been shown that a sex difference in response to thyroid feeding is present in rats (12). All of the rats received, *ad libitum*, a diet consisting of: Crisco, 4; casein, 20; salts, 4; cod liver oil (U.S.P.), 4; and starch, 62 parts. The amount of food eaten each day was measured. All rats received a daily supplement of 200 mg. of a dried baker's yeast which contained 23 I.U. of vitamin B_1, 60 μg. of riboflavin, 85 μg. of vitamin B_6, and 120 to 150 μg. of calcium pantothenate per gm. This amount of yeast will allow a gain in weight in the control rats, but will not prevent the typical loss of weight observed in thyroid-fed rats (3). The experimental groups received 100 mg. of desiccated thyroid gland per day. One of the thyroid-fed groups received, in addition to the 200 mg. of yeast, an additional 1 gm. of a yeast concentrate[3] per day, which contained 200 U.S.P. units of vitamin B_1, 230 μg. of riboflavin, 200 μg. of vitamin B_6, and 1500 to 2000 μg. of calcium pantothenate per gm. The thyroid gland and yeast supplements were fed to the control and to the test animals for 86 days. The thyroid-fed controls rapidly lost weight and were killed on the 47th day of the experiment. The rats, not fasted, were killed 24 hours after the last supplement of thyroid and yeast. Liver glycogen was determined by the method of Pfluger as modified by Good et al. (13) and glucose by the Hagedorn-Jenson method (14).

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[2] This work was done in the laboratory of Dr. W. W. Swingle and we are indebted to him for the necessary facilities to undertake this work.

[3] The authors are indebted to Dr. C. N. Fry of The Fleischmann Laboratories for the analyzed yeast, and to Dr. H. W. Rhodehamel of Eli Lilly & Co., Indianapolis, Ind. for the desiccated thyroid gland.

RESULTS

The control rats, fed 200 mg. of yeast per day, gained weight and showed normal liver glycogen values, the average being 2.51 ±0.36 per cent (table 1). The thyroid-fed control rats, receiving 200 mg. of yeast plus 100 mg. of thyroid gland per day, lost weight and showed a marked depletion of liver glycogen. However, the test rats, receiving 1 gm. of a yeast concentrate per day for 86 days in addition to the basal yeast and thyroid gland, showed no loss in weight and had an average liver

TABLE 1. EFFECT OF B VITAMINS ON LIVER GLYCOGEN DURING HYPERTHYROIDISM

Rat Number	Liver Glycogen, %	Per 100 gm. Rat		Weight of Animal		Comment
		Weight of liver, gm.	Glycogen in liver, mg.	At start	At end	
				of thyroid feeding, gm.		
Control animals						
241	2.98	3.1	92.4	281	296	
243	2.44	3.4	83.0	224	264	
245	2.12	3.6	76.3	216	218	
246	2.31	3.5	80.9	238	267	
250				194	187	Died
252	2.53	3.8	96.1	198	246	
285	2.47	3.5	86.5	209	215	
286	1.95	3.6	70.2	189	240	
287	3.25	3.2	104.0	190	217	
Av.	2.51±0.36	3.5±0.23	86.2±11.0	215	239	
Thyroid-fed controls						
234	0.32	3.5	11.2	224	187	
235	0.38	3.6	13.7	238	170	
236	0.32	4.1	13.1	198	140	
239	0.16	4.5	7.2	266	174	
242	0.27	3.9	10.5	204	174	
244	0.19	3.8	7.2	208	172	
284	0.44	3.7	16.3	188	107	
288	0.59	3.8	22.4	186	167	
289	0.43	4.1	17.6	174	161	
Av.	0.34±0.13	3.9±0.31	13.2±4.89	209	161	
Test animals						
247	2.41	5.0	120.5	236	238	Maintained weight
251	2.26	5.4	124.0	278	257	Lost weight
268	2.60	5.6	145.6	178	195	
272	2.28	5.2	118.6	184	191	
273	2.03	5.3	107.6	164	198	
274	1.87	5.5	102.9	206	194	Lost weight
276	2.12	5.1	108.1	189	198	
278	2.34	4.8	114.3	230	247	
301	1.76	5.2	91.5	171	160	Lost weight
308	2.17	5.7	123.7	185	184	Maintained weight
309	2.06	4.9	100.9	208	224	
310	3.04	5.3	161.1	195	231	
311				184	184	Died
312	2.09	5.4	112.9	180	199	
313	1.78	5.3	94.3	198	217	
Av.	2.20±0.34	5.3±0.25	116.1±20.9	199	208	

glycogen of 2.20 ± 0.34 per cent (table 1). This value is not statistically different from that of the control animals. Hence a yeast intake sufficient to prevent a loss of weight in hyperthyroid rats also enables the animals to maintain normal liver glycogen stores.

The amount of yeast fed, besides influencing the body weight and liver glycogen of the hyperthyroid rats, also affects the food intake of each group. The control rats ate an average of 12 gm. of food per day, and the thyroid-fed controls, receiving only the basal yeast supplement, consumed an average of 12 gm. of food per day. The voluntary food intake of the hyperthyroid rats receiving 1 gm. of yeast concentrate per day in addition to their basal supplement rose to an average of 18 gm. per day. This increased food intake in the test animals is probably the main factor in the maintenance of body weight and liver glycogen.

At autopsy it was observed that the weight of the liver of test animals was increased. When the liver weight was calculated on the basis of body weight, the increase in the weight of the liver of the test animals was found to be statistically significant over that of the control animals (table 1). The difference in the liver weight between the control animals and the thyroid-fed controls was not statistically significant. Calculations on basis of body weight also show that the amount of glycogen in the liver of the test rats is above that of the control rats (table 1). Although the difference between the means is not significant, the hypertrophy of the liver in the test animals will allow for a greater storage of glycogen.

DISCUSSION

Many theories have been advanced to explain the carbohydrate changes which occur during hyperthyroidism. The studies of Althausen and Stockholm (15) have clearly shown that intestinal phosphorylation and absorption of carbohydrates is increased during hyperthyroidism. It is also known that the utilization of dextrose is increased during hyperthyroidism and that the stores of glycogen in the liver are soon depleted. Sanger and Hun (16) believe that thyroxin exerts a toxic action on the liver, whereas Johnston (17) believes that it is the increased utilization of carbohydrate that decreases liver glycogen. Many authors have emphasized the relation of the pancreas to the carbohydrate disturbances seen in hyperthyroidism (cf. Althausen (18)). John (19) presents clinical evidence for this point of view. However, none of the above ideas accounts for all of the carbohydrate disturbances seen in clinical and experimental hyperthyroidism.

The field was clarified by Althausen and Stockholm (15), who found that intestinal phosphorylation and absorption of sugars was greatly increased during hyperthyroidism. This explains the high dextrose tolerance, the postprandial hyperglycemia, and the glycosuria seen in hyperthyroidism. Mirsky and Broh-Kahn (20) have also shown that hyperthyroidism increases the utilization of dextrose in the tissues of eviscerated animals. Thus, if the thyroid-fed animal does not compensate for the increased utilization of dextrose by a greater food intake, the liver glycogen will fall. The low liver glycogen will in turn cause, in the fasting animal, a low respiratory quotient. However, if the voluntary food intake of a hyperthyroid animal could be increased above normal it might be expected that the liver glycogen would be maintained at normal values. It has been shown that yeast, and more particularly vitamin B_1, greatly influences the food intake of thyroid fed animals. The administration of vitamin B_1 and yeast to hyperthyroid animals stimulates the food intake above normal and also allows maintenance of body weight. Thyroid feeding also decreases the amount of vitamin B_1 in rat tissues, particularly in the liver. Thus rats fed thyroid gland for 86 days, and receiving sufficient B vitamins in the form of yeast to stimulate

food intake and maintain body weight, also were able to maintain normal liver glycogen values.

Thyroid feeding would, therefore, seem to influence carbohydrate metabolism by the following mechanisms: *a*), by increasing the intestinal phosphorylation and absorption of carbohydrates; *b*), by increasing the utilization of dextrose by the tissues; *c*), by increasing the requirement of the animal for the B vitamins, and if the needed vitamins are not supplied in larger amounts a fall in liver glycogen will be obtained; *d*), supplying a larger intake of the B vitamins will increase the food intake, maintain body weight, and maintain the liver glycogen of the hyperthyroid animal at normal levels.

CONCLUSIONS

1. Hyperthyroid rats, receiving a small intake of the B vitamins, showed a marked loss of weight and a lowering of liver glycogen stores.

2. Hyperthyroid rats, receiving an increased amount of the B vitamins, sufficient to increase food intake and maintain body weight, had normal liver glycogen values after 86 days of thyroid feeding.

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COMPARATIVE GONADOTROPIN ASSAYS AND USE OF CALCIUM PHOSPHATE AS AN ADSORBENT IN CONCENTRATION OF FOLLICLE-STIMULATING ACTIVITY FROM PITUITARY GLAND[1]

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VERY SOON after the discovery of the gonadotropic activity of the pituitary with implants and extracts by Smith (1) and Zondek and Aschheim (2), it became obvious that the biological response was a complex one (3). At present, the most commonly accepted view is that two specific biological responses are involved, and that they are due to two specific substances (4), the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). The former causes follicular growth and maturation in the ovary, and spermatogenesis in the testis. The latter causes luteinization of the mature follicles in the ovary, and growth of the interstitial cells in the testis. Although the Evans (5) and the van Dyke (6) groups have reported the isolation of the luteinizing hormone in pure form, devoid of follicle-stimulating activity, nevertheless neither factor has been obtained in crystalline form.

The problem of separating and purifying the FSH and the LH factors is especially involved because of the difficulty of assaying each factor accurately when in a supposedly pure state and when mixed with other factors. Theoretically, hypophysectomized animals should be used in all of these assays and in particular in the testing of the final products. However, because of the cost and difficulty of preparing and maintaining large numbers of hypophysectomized animals, it was deemed expedient, in the case of preliminary fractionations, to use immature normal animals. Consequently, a number of comparative assays on glandular preparations were made with immature normal rats and mice, and in some cases with hypophysectomized rats.

MATERIALS ASSAYED

The pituitary products compared in these studies were acetone desiccated sheep pituitary powder and two concentrates therefrom, '*powder A*' and '*powder EB*'.

The acetone-desiccated sheep pituitary powder was obtained by repeatedly dehydrating finely ground, fresh, whole pituitary glands from sheep with acetone and finally removing the acetone from the tissue residue by means of a fan. The dried material was pulverized to a fine powder in a ball-mill or in a mortar.

Powder A (7) was prepared by adding 4 volumes of ethanol to a 0.02N ammonium-hydroxide extract of desiccated sheep pituitary powder. The extract was adjusted to pH 5.0 to 5.4 before the addition of the ethanol.

Powder EB was prepared from *powder A* by adsorption on tribasic calcium phosphate and elution with dilute alkali. The details of this method are reported later in this paper.

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METHODS OF ASSAY

Desiccated sheep pituitary powder and *powder A* were injected subcutaneously as suspensions and *powder EB* as a clear solution in physiological salt solution.

When immature normal female rats were used, the injections were given twice daily for 3 days. On the morning of the 4th day, the ovaries and uteri were carefully dissected, blotted with paper toweling, and weighed.

When immature normal male rats were used, the injections were given twice daily for 5 days. On the morning of the 6th day, the testes, seminal vesicles, anterior lobe of the prostate gland, and the adrenal glands were similarly removed and weighed.

The mouse uterine assay method was the same as that employed by J. S. Evans et al. (8) and similar to that of Hamburger and Pedersen-Bjergaard (9). Immature normal female mice were injected twice daily for 3 days. Thirty-six hours after the last injection, the uteri were removed, blotted with paper toweling, and weighed.

The hypophysectomized, immature male or female rats were injected twice daily for 4 days, and the autopsy was conducted on the 5th day. The injections were begun on the second day after the hypophysectomy. This period of time was not chosen arbitrarily, but was found by preliminary experiments to give more consistent results than if a longer rest period after operation were allowed. At autopsy, the top of the cranium was cut away, the brain carefully removed, and an examination made to ascertain whether the hypophysectomy was complete. Serial sections were not made. If the visual examination revealed an incomplete hypophysectomy, the organ weights of that animal were discarded.

In one experiment, *powder A* was assayed by the Marrian and Parkes method (10) in vitamin B_1-deficient young, mature, female rats. Fourteen young rats, 67 days old, were placed on a vitamin B_1-deficient diet consisting of 73 per cent starch, 20 per cent casein, 5 per cent inorganic salt mixture, and 2 per cent cod-liver oil. Within 4 weeks all of the rats developed a state of continuous anestrus. *Powder A* was then injected into these rats once daily for 3 days in an effort to produce a vaginal smear typical of estrus.

All of the rats were raised in our own colony. The mice were purchased from the Maple Grove Rabbitry of Springfield, Missouri.

The results of the assays are shown in tables 1 to 3 and figures 1 to 4. The organ weights are, in each case, average values compiled from one or more individual assays, each based on 5 or more animals.

DISCUSSION OF ASSAY RESULTS

The results of the vitamin B_1-deficiency experiment were as follows. The control rats remained in anestrus; the 3 rats injected with a total dose of 0.01 mg. of *powder A* also remained in anestrus. Two of 3 rats which received 0.1 mg. of *powder A* came into estrus within 4 days, and the 3 rats which received a total dose of 1.0 mg. gave vaginal smears typical of estrus within 2 days of the final injection. If no further injections were administered, the animals again lapsed into anestrus. All of the rats lost weight during the course of the experiment. When these deficient rats were returned to a complete diet, they gained weight rapidly, and when their body weights reached approximately the normal value for their age, the normal estrous cycle reappeared in every case.

It is evident that although the results of the vitamin B_1-deficiency experiment are in the proper order and confirm those of Marrian and Parkes (10), nevertheless such a method would be far too laborious and insensitive to be considered as a general assay method.

It is apparent from the results of the assays made in normal immature female rats (fig. 1), that there is a marked difference in the type of ovarian and uterine responses elicited by injections of *powder A* as compared with desiccated sheep pituitary powder. With *powder A* there occurred a sharp initial rise in the uterine response which tapered off in the vicinity of 50 to 70 mg. of uterine weight. The ovaries showed a gradual, almost linear, response until the weight reached 40 mg. whereupon the response remained on a plateau. On the other hand, the desiccated sheep pituitary powder (DSP) effected a prompt rise in uterine response up to a value of about 90 mg.

TABLE 1. EFFECT OF VARYING DOSES OF *powder A*, *powder EB*, AND DESICCATED SHEEP PITUITARY POWDER UPON THE OVARIAN, UTERINE, AND ADRENAL WEIGHTS OF HYPOPHYSECTOMIZED IMMATURE RATS[1]

Number of Rats Used	Total Dose Injected, mg.	Final Body Weight, gm.	Ovarian Weight, mg.	Uterine Weight, mg.	Adrenal Weight, mg.
		Powder A			
35	0.00	49	7.6	13.8	10.5
4	0.25	42	7.0	15.7	9.1
12	0.50	49	11.9	17.3	9.2
13	1.0	56	19.0	60.6	10.4
4	2.0	56	22.7	58.2	12.3
8	5.0	47	33.3	59.6	9.4
		Powder EB			
6	0.025	43	8.9	24.8	9.2
14	0.05	50	19.7	86.9	9.4
8	0.50	45	53.8	90.5	10.2
		Desiccated Sheep Pituitary Powder			
9	5.0	55	11.4	23.1	11.1
15	10.0	53	19.1	48.4	13.4

[1] Normal female rats, 34 days of age and weighing an average of 53 gm., were found to have an ovarian weight of 13.1 mg., a uterine weight of 20.5 mg., and an adrenal weight of 17.0 mg.

where a plateau resulted. The ovarian weight response was more gradual, but steadily upwards, so that even with the highest dose of desiccated sheep pituitary powder, the ovarian weight, which had already passed the 100-mg. level, was still ascending.

These results indicate that the desiccated sheep pituitary (DSP) powder may have contained some factor which was not present in *powder A* or that the ratio of luteinizing to follicle-stimulating hormone in the two powders was different. The latter explanation is more probable because the assays for the luteinizing activity in hypophysectomized male rats showed that *powder A* consists mainly of follicle-stimulating hormone.

In normal immature male rats (fig. 2), desiccated sheep pituitary powder (DSP), and *powder A*, respectively, produced very similar responses in the testes, but again some differences in the responses of the secondary sex organs (fig. 3). With *powder A* the response of the testes rose sharply with increased dosage and then maintained a plateau effect. The prostate weight response also rose sharply and then showed an irregular plateau effect. The weight of the seminal vesicles increased 50 per cent at once, but then remained at that level even when ten times the minimum dose necessary to effect the 50 per cent increase was administered. With desiccated sheep pituitary powder (DSP), the response of the testes was similar to that effected by

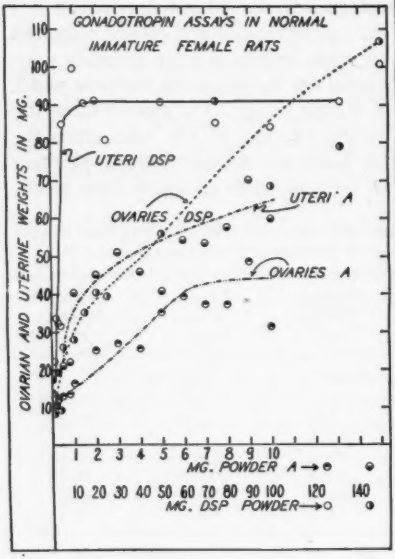


Fig. 1

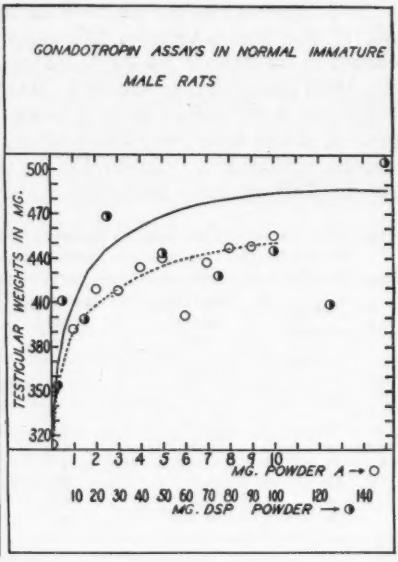


Fig. 2

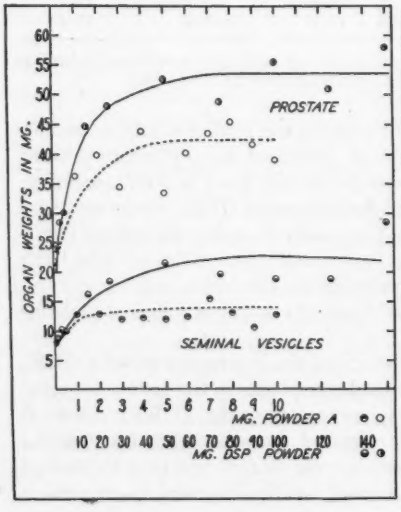


Fig. 3. Gonadotropin assays in normal immature male rats.

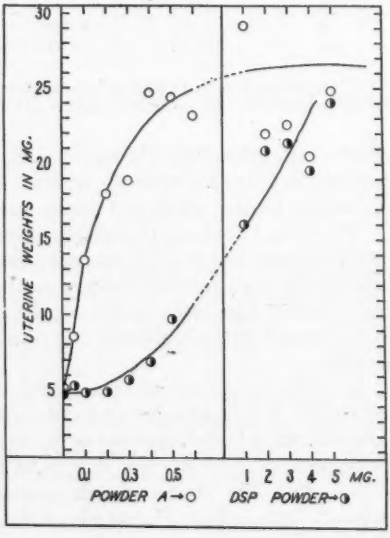


Fig. 4. Gonadotropin assays in normal immature female mice.

powder A. However, there were marked increases in the responses of the prostate and seminal vesicles as compared with the responses obtained with *powder A*. This increase in the weight of the seminal vesicles of normal immature male rats has been used by Fevold et al. (11) to test for luteinizing hormone.

These results again indicate that *powder A* is low in luteinizing activity. In all of our studies with normal immature rats, the adrenal weights remained at control values regardless of the dose of *powder A* or desiccated sheep pituitary powder. These results confirm the view that crude adrenotropic preparations cannot be assayed in normal immature rats.

The mouse uterine assay method yielded very consistent results (fig. 4) and proved

TABLE 2. EFFECT OF VARYING DOSES OF *powder A*, DESICCATED SHEEP PITUITARY POWDER, AND *powder EB* UPON WEIGHTS OF TESTES, SEMINAL VESICLES, ANTERIOR LOBE OF PROSTATE GLAND, AND ADRENAL GLANDS OF HYPOPHYSECTOMIZED IMMATURE RATS[1]

Total Dose Injected, mg.	Final Body Weight, gm.	Testes mg.	Seminal Vesicles, mg.	Prostate Gland, mg.	Adrenal Glands, mg.
		Powder A			
0.0	47 (30)[2]	157	6.3	7.4	9.5
1.0	41 (5)	274	9.9	20.7	7.2
5.0	45 (5)	299	6.7	17.2	10.2
		Powder EB			
0.05	49 (7)	355	7.1	12.3	8.4
0.10	42 (5)	334	6.1	18.7	8.4
0.50	41 (3)	273	5.8	16.6	8.9
		Desiccated Sheep Pituitary Powder			
5.0	46 (5)	225	8.6	18.0	10.9
10.0	47 (7)	252	11.4	32.9	10.5

[1] Normal male rats, 34 days of age and weighing an average of 47 gm., were found to have a testicular weight of 268 mg., a seminal vesicle weight of 7.0 mg., a prostate weight of 19.1 mg., and an adrenal weight of 17.3 mg.

[2] The figures in parentheses designate the number of animals used.

to be the most sensitive method for measuring follicle-stimulating activity. This method of assay indirectly measures the effect of the follicle-stimulating principle through the secretion of estrogens by the follicles. The results of the assays showed desiccated sheep pituitary powder (DSP) to be about one-tenth as active, weight for weight, as *powder A*. The responses obtained varied slightly with the temperature of the environment, for it was found that the uterine response was about 20 per cent higher during the hot months than during the cold months.

Powder EB was found to be very active by the mouse uterine method, as little as 0.006 mg. causing a 50 to 100 per cent increase in the uterine weight.

These results confirm the earlier reports (8) that the immature mouse uterine weight change is a sensitive assay method for the follicle-stimulating principle. The data show that it is 5 to 10 times as sensitive as the method involving hypophysectomized immature female rats and 20 times as sensitive as the method involving normal immature female rats.

The results of the assays on hypophysectomized rats (table 1) are more significant than the results of assays in which animals with intact pituitary glands are employed,

because the intact pituitary gland of the animal may seriously modify the character of the response. The assays on hypophysectomized female rats indicate that the follicle-stimulating activity in *powder EB* is about 20 times as concentrated as in *powder A* and about 200 times as concentrated as in desiccated sheep pituitary powder.

The hypophysectomized male rats were used to measure the luteinizing activity in terms of the increase in the weight of the prostate gland (table 2). It must however be pointed out that the results on hypophysectomized male rats were based upon fewer animals than the results on hypophysectomized female rats. The results appear

TABLE 3. COMPARISON OF RESULTS OBTAINED FROM ASSAYS ON DESICCATED SHEEP PITUITARY POWDER *powder A*, AND *powder EB*

Material Assayed	FSH Assays	LH Assays
	A) In hypophysectomized female rats	In hypophysectomized male rats
	B) In normal immature female mice	
Acetone-desiccated sheep pituitary powder	A) >5.0 mg.<10.0 mg.=1 R.U.	5.0 mg.≧1 R.U.; however, a 10.0 mg. dose effected an 80% weight increase in seminal vesicles and a 300% weight increase in the prostate
	B) 0.5 mg.=1 M.U.	
Powder A (5% by weight yield from desiccated sheep pituitary powder)	A) >0.5 mg.<1.0 mg.=1 R.U.	1.0 mg.≧1 R.U., a 5.0-mg. dose caused no further increase in prostatic weight; seminal vesicles were in neither case any larger than those of the controls
	B) 0.05 mg.=1 M.U.	
Powder EB (1.5–2.0% by weight yield from *powder A*)	A) >0.025 mg.<0.05 mg.=1 R.U.	0.1 mg.≧1 R.U.; a 0.5-mg. dose effected no further increase in prostatic weight; seminal vesicles were in neither case any larger than the controls
	B) 0.006 mg.=1 M.U.	

100% increase in rat ovarian weight=1 R.U. of FSH.
100% increase in rat prostatic weight=1 R.U. of LH.
50–100% increase in mouse uterine weight=1 M.U. of FSH.

to be clear-cut with the desiccated sheep pituitary powder, for the responses parallel the doses. However, with *powder A* and especially with *powder EB*, the results of the assays are questionable. Although a definite prostate response was elicited in both cases, nevertheless, when 5 times this minimal effective dose was administered, there was no better prostate response, but instead a slightly lower one. Furthermore, although the seminal vesicles increased in weight with desiccated sheep pituitary powder, especially at higher doses, they increased only slightly with *powder A* and not at all when *powder EB* was administered. That *powder EB* has little or no luteinizing activity was substantiated by the histological sections of ovaries from hypophysectomized rats to which the highest doses of *powder EB* had been administered (fig. 5). Microscopic examination of these sections showed numerous follicles and almost no corpora lutea.

Desiccated sheep pituitary powder was the only preparation which caused a weight increase in the adrenal glands of hypophysectomized male and female rats. This indicates that neither *powder A* nor *powder EB* contains any appreciable adrenotropic activity. Furthermore, a few isolated assays in one-day-old cockerels showed that desiccated sheep pituitary powder contains a small, but definite, amount of thyrotropic activity, but that *powder A* contains very little and *powder EB* no thyrotropic activity.

The relative activities of desiccated sheep pituitary powder, *powder A* and *powder EB*, as interpreted from our data, are presented in table 3. Since standard definitions of units for the follicle-stimulating and the luteinizing hormones have not been adopted, we have arbitrarily assigned the units indicated. From the data presented in table 3, it is apparent that on the basis of the mouse assays, *powder EB* is about 9 times as potent as *powder A* and about 90 times as potent as desiccated sheep pituitary powder with respect to follicle-stimulating activity. On the basis of the assays in hypophysectomized female rats, *powder EB* is 20 times as active as *powder A* and 200 times as active as desiccated sheep pituitary powder. The results obtained on the hypophysectomized male rat, as stated previously, are of questionable value except in the case of desiccated sheep pituitary powder.

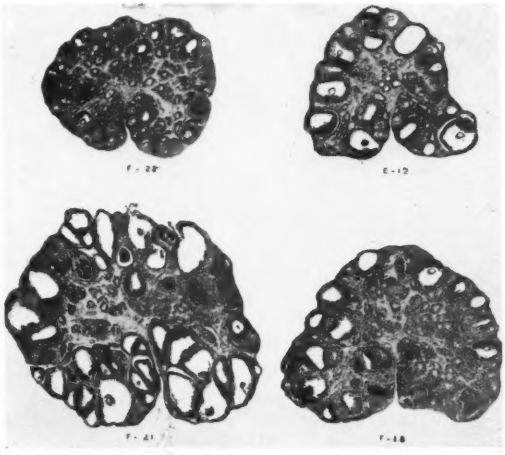


Fig. 5. OVARIES FROM HYPOPHYSECTOMIZED RATS TREATED WITH PITUITARY PREPARATIONS. *Section E-12* shows the effect from 0.05 mg. of *powder EB; section F-21*, 0.50 mg. of *powder EB; section F-18*, 10.0 mg. of desiccated sheep pituitary powder; *section F-22*, control.

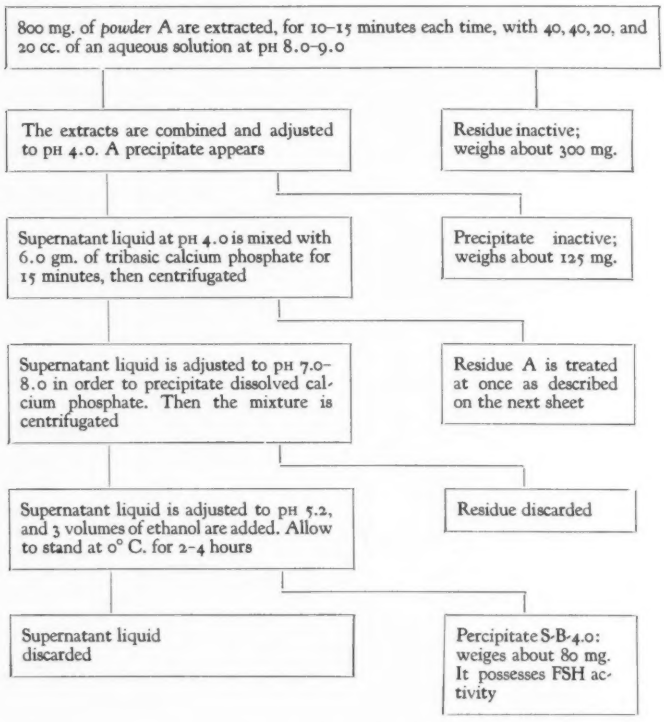
Preparation of Powder EB

Reports in the literature on the separation of the luteinizing (LH) and the follicle-stimulating (FSH) hormones are limited to the use of proteolytic enzymes (12, 13) and to fractional precipitation by alcohol and salts, especially ammonium sulphate (11, 14, 15, 16). No study involving the use of adsorbents seems to have been reported. In this paper are presented the results obtained with the use of adsorbents.

The starting product for these experiments was *powder A*. Preliminary adsorption studies upon aqueous extracts of *powder A* with tribasic calcium phosphate (Merck), Lloyd's reagent (a form of hydrated aluminum silicate), alumina III (aluminum oxide heated to 150°C. for 2 hours), permutit (an artificially prepared aluminum silicate, activated with dilute acetic acid), supercel (essentially silicon dioxide), aluminum hydroxide cream (a colloidal form of aluminum hydroxide used by Hektoen and Welker (17) in their antigen studies), and magnesium oxide, indicated that the last three were useless for concentrating the follicle-stimulating activity under the conditions of our studies. The other four substances all showed definite promise of being of value, but tribasic calcium phosphate (Merck) proved most effective. The details of the ex-

perimental procedure finally adopted, and the yields of the various fractions obtained, are shown on flow sheets 1 and 2.

FLOW SHEET 1. OUTLINE OF THE ADSORPTION PROCEDURE

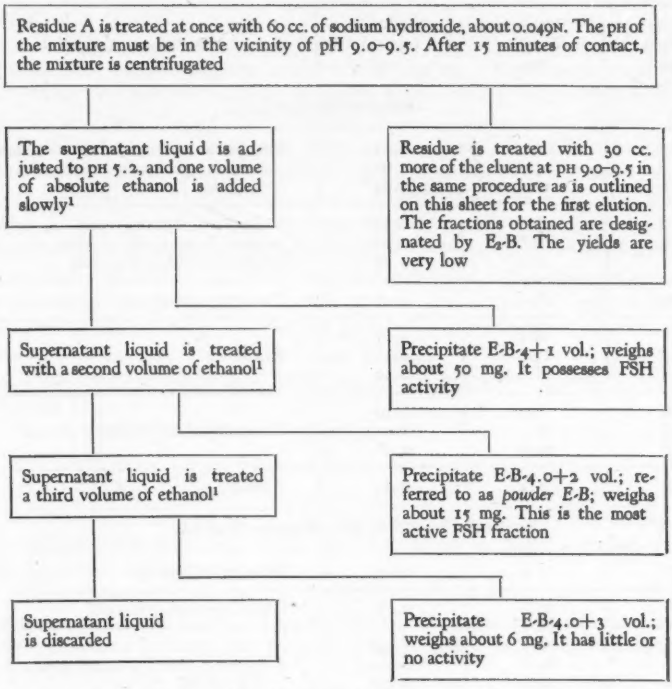

800 mg. of *powder* A are extracted, for 10–15 minutes each time, with 40, 40, 20, and 20 cc. of an aqueous solution at pH 8.0–9.0
The extracts are combined and adjusted to pH 4.0. A precipitate appears
Residue inactive; weighs about 300 mg.
Supernatant liquid at pH 4.0 is mixed with 6.0 gm. of tribasic calcium phosphate for 15 minutes, then centrifugated
Precipitate inactive; weighs about 125 mg.
Supernatant liquid is adjusted to pH 7.0–8.0 in order to precipitate dissolved calcium phosphate. Then the mixture is centrifugated
Residue A is treated at once as described on the next sheet
Supernatant liquid is adjusted to pH 5.2, and 3 volumes of ethanol are added. Allow to stand at 0° C. for 2–4 hours
Residue discarded
Supernatant liquid discarded
Percipitate S-B-4.0: weiges about 80 mg. It possesses FSH activity

The optimum pH for eluting the follicle-stimulating activity was in the vicinity of pH 9.0 to 9.5. If the pH was maintained above 10.0 during the elution, some destruction of follicle-stimulating activity occurred. If the pH exceeded 11.0, the activity was entirely destroyed, and upon acidification, prior to the addition of alcohol, an odor, similar to that of hydrogen sulphide could be detected. If, during elution, the pH remained below 8.5, the activity was only slowly and partially removed. If the pH was maintained below 7.5, no activity was eluted. A weakly alkaline solution (0.049N sodium hydroxide), adjusted, during the course of elution, to pH 9.0 to 9.5, was as effective as a glycine-sodium hydroxide buffer in eluting the follicle-stimulating activity.

When a 0.02N ammonium hydroxide extract of desiccated sheep pituitary powder was used in place of the aqueous extract of *powder A* as shown in flow sheet 1, the activity and yields of the various fractions were low. This indicates that in the precipitation of *powder A* by alcohol from a 0.02N ammonium hydroxide extract of desiccated sheep pituitary powder (pH adjusted to 5.0 to 5.4 before alcohol addition), some interfering substances are removed.

A number of attempts were made to use a column of calcium phosphate for the adsorption of the follicle-stimulating activity. However, the procedures were too slow and led to very irregular results.

FLOW SHEET 2


Residue A is treated at once with 60 cc. of sodium hydroxide, about 0.049N. The pH of the mixture must be in the vicinity of pH 9.0–9.5. After 15 minutes of contact, the mixture is centrifugated
The supernatant liquid is adjusted to pH 5.2, and one volume of absolute ethanol is added slowly[1]
Residue is treated with 30 cc. more of the eluent at pH 9.0–9.5 in the same procedure as is outlined on this sheet for the first elution. The fractions obtained are designated by E2-B. The yields are very low
Supernatant liquid is treated with a second volume of ethanol[1]
Precipitate E-B-4+1 vol.; weighs about 50 mg. It possesses FSH activity
Supernatant liquid is treated a third volume of ethanol[1]
Precipitate E-B-4.0+2 vol.; referred to as powder E-B; weighs about 15 mg. This is the most active FSH fraction
Supernatant liquid is discarded
Precipitate E-B-4.0+3 vol.; weighs about 6 mg. It has little or no activity

[1] The mixture is allowed to stand at 0° C. for a minimum of several hours to facilitate the precipitation.

In several experiments, extracts of *powder A* and solutions of *powder EB* were fractionated with ammonium sulphate by the McMeekin procedure (18) as reported by Fevold et al. (11) except that in some of the experiments, a capillary tube was substituted for the cellophane membrane as the means of introducing the salt. This modification was made in order to lessen the time needed to introduce the ammonium sulphate.

EXPERIMENTAL RESULTS

The gonadotropic activities of the original product and the various actions secured according to flow sheets 1 and 2, and by ammonium sulphate precipitation, are indicated in table 4. These results show that on the addition of the second volume of ethanol to the eluted material, the most potent follicle-stimulating fraction was obtained. This fraction, referred to as *powder EB*, was also assayed in hypophysectomized male and female rats (tables 1 and 2), and the results of those assays have already been discussed.

From the data presented in table 4, it appears that about 50 to 60 per cent of the total follicle-stimulating activity in *powder A* is recovered in the various fractions obtained by means of the adsorption procedure. About one-third of this recovered FSH activity resides in *powder EB*. The data also indicate that *powder EB* is about 3 to 4 times as active as the most potent fraction obtained from an extract of *powder A* by fractional precipitation with ethanol.

TABLE 4. MOUSE ASSAY RESULTS

Body Weight, gm.	Uterine Weight, mg.	Total Dose Injected, mg.	Substance Assayed
		Reference Substances	
9	6.1	0.012	Precipitate obtained when 1 volume of alcohol was added to an extract of *powder A*; 17% yield
9	15.8	0.025	
8	18.5	0.05	
8	6.1	0.012	Precipitate obtained from an extract of *powder A* upon the addition of a second volume of alcohol; 5% yield
8	8.6	0.025	
8	16.3	0.05	
10	6.1	0.05	*Powder A* extract+3rd volume; 2% yield
8	8.5	0.05	*Powder A*
8	13.6	0.10	
8	18.0	0.20	
8	9.7	0.50	Desiccated sheep pituitary powder
8	16.0	1.00	
8	20.9	2.00	
8	5.1	0.00	Controls
		Fractions Obtained by the Adsorption Procedure	
7	11.4	0.05	S-B-4.0; 10% yield
8	18.8	0.05	E-B-4.0+1 vol.; 6% yield
7	8.7	0.006	E-B-4.0+2 vol.; 1.5–2.0% yield (*Powder EB*)
9	19.8	0.012	
8	24.2	0.025	
8	5.1	0.05	E-B-4.0+3 vol.; <1% yield
7	11.3	0.05	E_2-B-4.0+1 vol.; 4% yield
8	7.5	0.012	E_2-B-4.0+2 vol.; 0.5% yield
7	14.1	0.025	
		Fractions Obtained by the Ammonium Sulphate Method	
10	8.7	0.10	LH fraction obtained at 2.1M ammonium sulphate concentration; 21% yield
8	9.8	0.15	
9	11.7	0.33	
9	17.9	0.07	FSH fraction obtained at 3.1M ammonium sulphate concentration; 15% yield
9	18.9	0.13	
11	23.9	0.27	

Measured by the mouse assays, the recovery of the total follicle-stimulating activity by precipitation with ammonium sulphate was of the same order as by our adsorption method. However, the LH fraction obtained at 2.1M ammonium sulphate concentration appeared to have definite follicle-stimulating activity, and the FSH fraction precipitated at 3.1M ammonium sulphate concentration, although of good follicle-stimulating activity, was far less potent than *powder EB*. Several assays in hypophysectomized rats also indicated that the separation of the luteinizing and follicle-stimulating activities was incomplete by the ammonium sulphate precipitation.

Chemical Tests

An attempt to make a quantitative estimation of glycuronic acid by the naphthoresorcinol reaction led to negative results on *powder A* and *powder EB*.[2]

Qualitative protein reactions were carried out on a 1 per cent suspension of *powder A* and a 0.1 per cent solution of *powder EB* in parallel with a 1 per cent casein suspension used as a control. The Molisch, biuret, xanthoproteic, Millon, Ehrlich Diazo, Vanillin, and reduced sulphur tests were positive. The reduced sulphur test was much more strongly positive with the 1 per cent *powder A* suspension than with the 1 per cent casein. Although the 0.1 per cent solution of *powder EB* gave a positive test for reduced sulphur, it was much less intense than with the 1 per cent *powder A* suspension. This test for reduced sulphur appears significant, for, as stated previously, when too basic an eluting agent was used to remove the follicle-stimulating activity adsorbed on calcium phosphate, the activity was destroyed, and upon subsequent acidification of the mixture, an odor similar to that of hydrogen sulphide could be detected. This supports the view of Fraenkel-Conrat, Simpson, and Evans (19) who reported that treating the FSH or ICSH fraction with cysteine resulted in inactivation in both cases.

SUMMARY

1. The sensitivity and reliability of the immature mouse-uterine-weight response as an assay procedure for the follicle-stimulating hormone was confirmed. It was the most sensitive method tried, being 5 to 10 times more sensitive than the hypophysectomized immature-female-rat test and about 20 times more sensitive than the normal immature-female-rat test.

2. Hypophysectomized immature female rats proved to be the most specific for the assay of the follicle-stimulating principle of the pituitary. Hypophysectomized immature male rats were superior to normal intact male rats for the specific determination of the luteinizing hormone.

3. Adsorption of the follicle-stimulating activity from *powder A* on tricalcium phosphate, elution with dilute sodium hydroxide, and fractional precipitation of the eluted activity with ethanol, yielded a very potent follicle-stimulating fraction, *powder EB*.

4. On the basis of comparative assays in hypophysectomized female rats, *powder EB* was found to be 20 times as potent as *powder A* and 200 times as potent as desiccated sheep pituitary powder in follicle-stimulating activity. *Powder EB* was also found to be more potent as a follicle-stimulating preparation than those obtained by fractionation with ammonium sulphate.

5. At the levels assayed, *powder EB* gave negative adrenotropic and thyrotropic assays, and the presence of any appreciable amount of luteinizing activity was questionable.

6. *Powder EB* as well as *powder A* were found to be free of glycuronic acid, but gave positive responses to the common protein and amino acid tests, especially the reduced sulphur test.

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PITUITARY WEIGHT OF GROWING MALE ALBINO RAT RELATED TO BODY WEIGHT[1]

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HATAI (1) has reported on the relation of the weights of various organs (including the pituitary) of the growing albino rat to body weight. Others (2–6) have similarly reported on such relations in the mature albino rat. For more complete references to relation between organ weight and body weight in growing and mature animals see Brody and Kibler (7).

Since the time of Hatai's report on the albino rat the pituitary gland has become increasingly recognized as an important organ of internal secretion, being associated with many body processes and with the growth of the animal. As the pituitary is so closely associated with so many vital processes in the body, it has been suggested (8) that the relation between increasing pituitary weight and increasing body weight be used as a criterion for judging amounts of hormone or hormone extracts to administer to animals of different body weights which are used in endocrine research. For instance, the investigator who wishes to maintain normal growth rates in hypophysectomized animals with pituitary growth extracts has a definite problem of adjusting the dosage level of extract injected to the increasing body weight of the experimental animal. Such increases in dosage level might parallel the increase in pituitary weight with increasing body weight. It is with these thoughts in mind, that these additional data on the relation of body weight to pituitary weight in the male albino rat are presented.

PROCEDURE AND ANALYSIS OF DATA

One hundred and thirty-five albino rats ranging in body weight from 60 to 383 gm. were used in this study. These animals were from a colony of Wistar Institute strain of albino rats, secured from the Department of Agricultural Chemistry through the courtesy of Dr. A. G. Hogan. These animals had been fed a complete grain mixture, had been kept at ordinary room temperatures and were killed in January.

The animals were killed with ether anesthesia, body weights were recorded, and the whole pituitaries were removed and weighed immediately to the closest 0.1 mg.

The coefficient of correlation between the body weights, X, and the pituitary weights, Y, of these rats was computed with the use of a frequency table according to the Pearson Product Moment Method as presented by Holzinger (9). In this frequency table the body weights were grouped into 30-gm. classes starting with the 60-gm. weight while the pituitary weights were grouped in 1-mg. classes. A coefficient of correlation, r_{xy}, of $+.92 \pm .01$ (P.E.) was secured, showing that a high degree of positive correlation exists between these two factors. A linear regression equation

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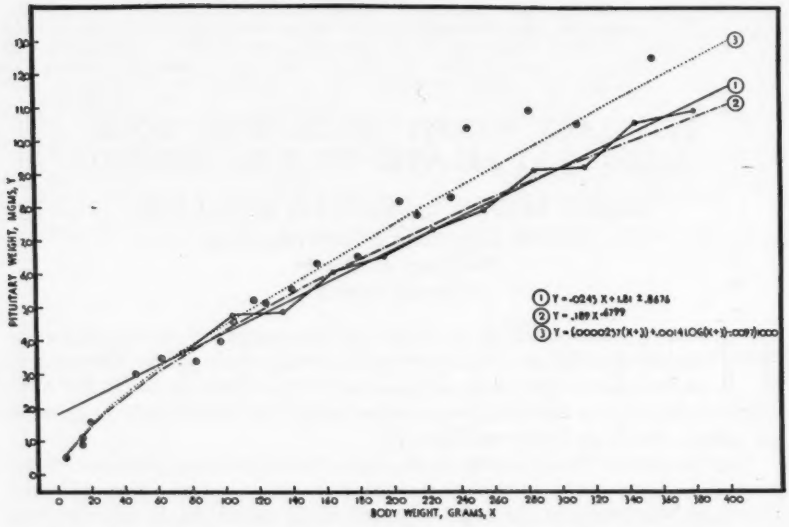

Fig. 1. WEIGHT OF PITUITARY of the male albino rat according to body weight. The observed weights of equations 1 and 2 are represented by 135 rats. The observed weights of equation 3 by Hatai (1) are represented by 78 rats. Linear equation 1 was fitted by the Product Moment Method to the general equation $Y = a + bX$. Expontential equation 2 was derived by fitting the logarithms of the line of mean values by the method of least squares to the formula $Y = aX^b$. Mean Pituitary Weights for various body weight groups, circles. Line of means on regression line 1, circles connected by lines. Observed mean weights by Hatai, circles with dot in center.

of the form $Y = a + bX$ was fitted to the data according to the formula and procedure as outlined by Holzinger (9). This regression line number 1

(1) $$Y \text{ (pituitary weight)} = .0245X \text{ (body weight)} + 1.81 \pm .87$$

together with a line of means as calculated from the frequency table is presented in figure 1. This equation may be used to predict the pituitary weights of male albino rats weighing between 60 and 390 gm. if the body weights are known.

Brody and Kibler (7) in their analyses of organ and body weights have plotted organ weights against body weight on logarithmic paper, fitting the data by the method of least squares to the relative growth equation, $Y = aX^b$. Using this technic the coordinate values as represented by the points in the 'line of means' (see figure 1) were fitted to this general equation. This regression line number 2 is also presented

(2) $$Y \text{ (pituitary weight)} = .189X^{.68} \text{ (body weight)}$$

in figure 1. Regression line number 2 is especially interesting when compared to regression line number 1 and also when both are compared to the original regression line for male albino rats as presented by Hatai (1) and shown as regression line number 3 in figure 1. The circles with a dot in the center of each in figure 1 represent the data points on which Hatai's regression line is based.

Figure 1 indicates that there may be a significant difference in the weights of the pituitaries of Hatai's rats and the rats here reported. Hatai's pituitary weights on Wistar Institute male albino rats observed over 30 years ago appear to be significantly heavier than those of this present day strain of Wistar animals.

Regression equation number 2 can probably best be used to predict the pituitary

TABLE 1. DERIVATION FROM EQUATION NUMBER 2 OF PITUITARY WEIGHTS AND RELATIONS IN GROWING MALE ALBINO RATS

Body Weight	Pituitary Weight	Pituitary Weight / Body Weight	Increase	
			In Body Wt.	In Pituitary Wt. or Injection Dosage
gm.	mg.	%	%	%
5	.56	.01120	1	.68
10	.90	.00900	5	3.4
20	1.45	.00725	10	6.7
30	1.91	.00636	20	13.2
40	2.32	.00580	30	19.8
50	2.70	.00540	40	25.7
60	3.06	.00510	50	31.7
80	3.72	.00465	60	37.6
100	4.33	.00433	70	43.8
150	5.70	.00380	80	49.1
200	6.93	.00347	90	54.7
250	8.07	.00323	100	60.2
300	9.13	.00304		
350	10.14	.00290		
400	11.10	.00278		

weights of male albino rats from birth up to 400 gm. in weight (see table 1) as shown by its closeness of fit to the data points. This equation indicates that pituitary weight is not directly proportional to body weight but varies with the .68 power of body weight since the exponent *b* is .68, and the ratio of pituitary weight to body weight decreases as body weight increases (see table 1). From table 1 it may be seen that an increase of 100 per cent in body weight causes an increase of only 60.2 per cent in pituitary weight while an incresae of 1 per cent in body weight causes a .68 per cent increase in pituitary weight which figure is identical with the value of exponent b in equation 2. As was indicated before, it is this increase in pituitary weight with increasing body weight which might be used as a basis for computing dosages.

SUMMARY

Data relating pituitary weight and body weights of male albino rats (Wistar strain) were correlated by *a*), fitting the data by the Product Moment Method to the equation $Y = a + bX$; and by *b*), fitting the points on the line of means by the method of least squares to the equation $Y = aX^b$. By the use of these equations or the table of values, pituitary weights may be predicted if body weights are known.

It is suggested that hormone injection in male albino rats (particularly pituitary injections or hormones which stimulate the pituitary) be varied with increasing body weights in proportion to the rate at which pituitary weight increases with body weight.

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PANCREATIC DIABETES IN THE MONKEY

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ALTHOUGH THE MONKEY has been used for many studies relative to the physiology of man, no systematic report exists in the literature with reference to pancreatic diabetes in this species. In 1937, Collip, Selye and Neufeld (1) reported that the monkey can survive pancreatectomy for many months without insulin treatment; that the ketonuria which may be observed during the first few days following the operation disappears later irrespective of whether the animal was treated; that the depancreatized monkey shows a marked sensitivity to insulin and rapidly develops hypoglycemia on fasting, and finally that such animals lose weight rapidly. Similar observations were made by Chapman and Fulton (2) who concluded that, unlike depancreatized carnivora, depancreatized monkeys do not die in typical diabetic acidosis. Both groups of investigators express the opinion that the depancreatized monkey exhibited many of the metabolic characteristics of the depancreatized-hypophysectomized dog. In 1941, we reported some preliminary studies on the development of acidosis and coma in the rhesus monkey and emphasized that acetonemia follows rapidly upon the withdrawal of insulin and food and that this acetonemia is accompanied by a decrease in the carbon dioxide combining power and a decrease in the pH of the blood (3). These changes were associated with the typical symptoms observed in man, such as weakness, dehydration, Kussmaul breathing, and tarry vomitus, and that when acetonemia reached levels of 175 mg. per cent or over, the animals became lethargic and, unless treatment was instituted, coma ensued.

Because of this remarkable resemblance to the syndrome of diabetic acidosis observed in man, it became of interest to observe the sequence of events that may occur in the depancreatized monkey maintained with insulin over a long period of time.

METHOD

Macacus rhesus monkeys were maintained in the laboratory on normal diet for several weeks before operation. Pancreatectomy was performed under nembutal anesthesia. In several instances in which the animal was permitted to die in coma, sections were made of tissues taken from the region of the pancrease to ascertain complete removal of this organ. Insulin[2] therapy was instituted within a few hours after the operation and within 24 hours thereafter the animals were allowed food ad libitum, and records were kept of the daily intake. The diet consisted of oranges, bananas and biscuits. The biscuits were composed of a mixture of 20 parts dog ra-

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[2] We are indebted to the Eli Lilly & Co. for generous supplies of insulin.

tion,[3] 1 part pancreatin,[4] and 4 parts ground raw peanuts. This diet was supplemented with adequate amounts of vitamin A and D concentrate. The daily insulin dosage was regulated so as to permit a urinary excretion of from 5 to 15 gm. of glucose per day. No experiments on the effect of insulin or of food withdrawal were made until the weight of the animal had been stabilized for several weeks. The sugar and acetone body concentration of the blood was determined on samples taken in the morning while the urinary excretion of these substances and of nitrogen was measured daily.

RESULTS

The operative wounds healed very rapidly and within 2 weeks the animals had, to all intents, recovered. Within a few days after the operation the weight of the

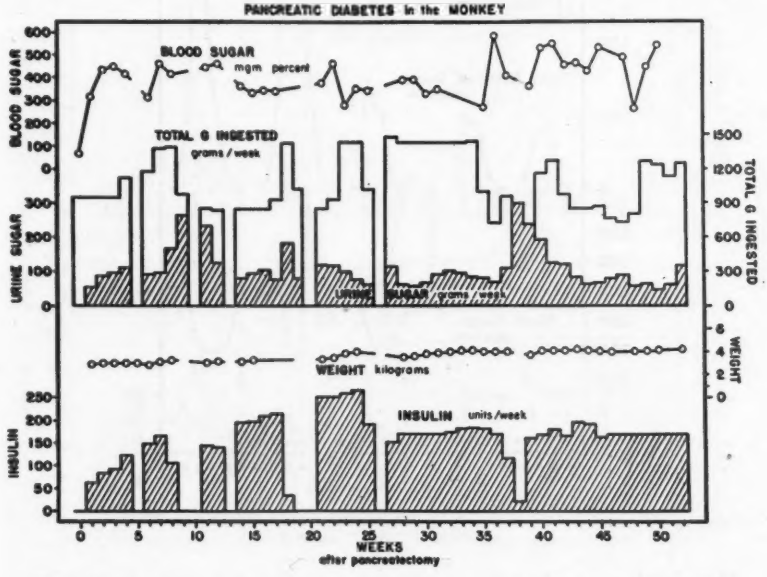

Fig. 1. Data obtained in a depancreatized monkey over a period of 52 weeks. The findings during the intervals in which exogenous insulin was stopped are not depicted.

animals returned to the preoperative level. Throughout the period of study the weight remained constant or gradually increased. No abnormalities in behavior were noted in the depancreatized monkeys as compared with the normal monkeys. After several weeks of insulin administration the animals became quite accustomed to handling and subjected themselves freely to the subcutaneous injections.

Figure 1 illustrates the observations in a monkey studied for a period of more than 50 weeks after pancreatectomy. It may be noted that it took approximately 10 weeks to establish the dose of insulin which maintained the animals at the desired level of glucose excretion. Although it is possible that this animal's diabetic severity increased up to that time, it is more probable that the dietary intake was the re-

[3] Dog ration consisted of 40.8 per cent carbohydrate, 22.3 per cent protein and 4.5 per cent fat, prepared by the Ubiko Mills.

[4] We are indebted to Dr. D. Klein of the Wilson Co. for generous supplies of pancreatin.

sponsible factor. The animal had a fairly constant intake for approximately 30 weeks and gained almost 2 kg. in body weight. The intake subsequently was decreased and the body weight returned to the original level. Throughout the period studied, ketonuria never occurred except at those times when insulin or food was discontinued. It may be noted that the blood sugar fluctuated between 200 and 400 mg. per cent, the preoperative level being from 60 to 100 mg. per cent.

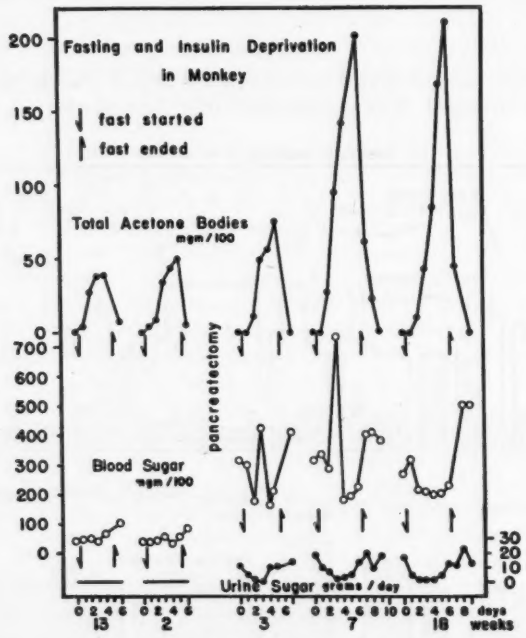

Fig. 2. INFLUENCE OF FOOD AND INSULIN DEPRIVATION of the depancreatized monkey depicted in figure 1.

Figure 2 illustrates the influence of fasting and of insulin deprivation in this monkey. During the control phase, before this monkey was depancreatized, fasting resulted in no signixcant change in the blood sugar, but did result in the development of an acetonemia which reached a maximum of 50 mg. per cent. After pancreatectomy, fasting and insulin deprivation always resulted in fluctuations in both the blood sugar and urinary excretion of sugar. The first response to fasting was a drop in the blood sugar which subsequently increased and then receded. The fluctuations in blood sugar consequent to insulin deprivation varied from low levels of 150 to high levels of 730 mg. per cent, dependent upon the intake of food. In all instances the urinary sugar fell. However, in no period did the blood sugar reach hypoglycemic stages. Associated with these fluctuations in the blood sugar and with the decrease in urinary excretion was a marked acetonemia which reached levels as high as 211 mg. per cent. In this animal, when the ketonemia was 169 mg. per cent, the CO_2 content of the blood fell to 22.4 volumes per cent, and the symptoms of acidosis, Kussmaul breathing, dehydration and lethargy were quite obvious. When the acetonemia reached a level of 211 mg. per cent, at which time the CO_2 was 23.2 vol. per cent, the animal was comatose.

In this state the animal could not be aroused, exhibited cold, clammy extremities, cyanosis and dehydration of the eye balls, gums and tongue. The administration of glucose intravenously together with insulin and subsequently the administration of orange juice by mouth and the repeated injection of insulin aroused the animal sufficiently to permit him to eat. Within 18 hours the animal was very markedly improved and in 24 hours seemed to have recovered completely from the episode of acidosis. This syndrome could be induced at will, and two such episodes are demonstrated in figure 2. The ratio of the amounts of dextrose to nitrogen excreted in the urine (D:N) during insulin deprivation was 3.5:1.

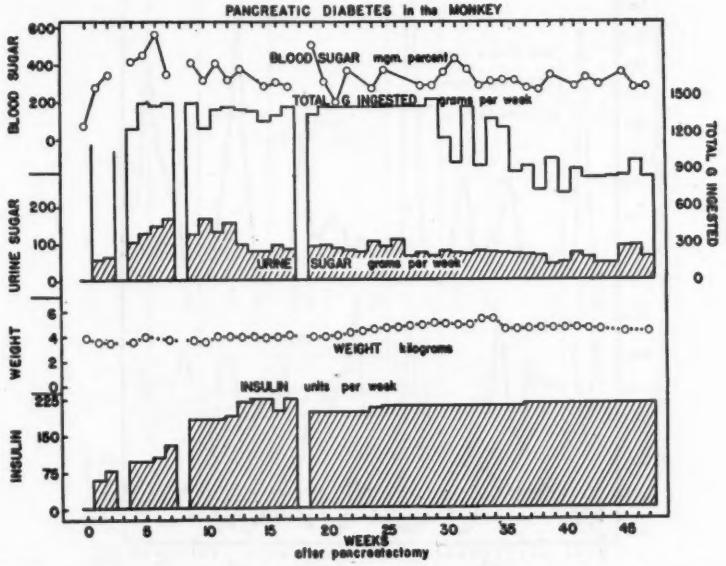

Fig. 3. DATA OBTAINED IN DEPANCREATIZED MONKEY for 47 weeks after pancreatectomy. The periods during which exogenous insulin was not administered are not depicted.

Figure 3 is illustrative of the history of another monkey which was treated in the same manner as that described above, and developed an identical picture.

Figure 4 illustrates the effects of insulin and food deprivation in this monkey and it may be noted that when both food and insulin were stopped (5th week after pancreatectomy), a severe acetonemia ensued within 48 hours. As previously described, the rising acetonemia was associated with weakness, dehydration, Kussmaul breathing and on occasion tarry vomitus. At the height of the acetonemia the animal was in coma and could not be aroused. The institution of insulin and glucose therapy resulted in a rapid recovery so that within 48 hours the animal was apparently normal. In a second period of insulin deprivation (9th and 10th week after pancreatectomy) the animal was permitted to eat and it may be noted that not only was the acetonemia less severe, but the rate at which the acetonemia increased was much slower; in some instances it began to decrease even though insulin was not administered. When the food also was stopped after 11 days of insulin deprivation, the acetonemia immediately rose again. Of interest is the fact that when the animal ate during this period of insulin deprivation, the glycosuria was maintained, but when food was withdrawn

the glycosuria diminished markedly and even completely disappeared. The restitution of food and insulin resulted in a return of the animal to its previous apparent normal state. In the 13th week after pancreatectomy, insulin and food were again eliminated and again a rapid acetonemia ensued so that in 48 hours the animal was in coma and the blood acetonemia was 227 mg. per cent, the CO_2 8.8 vol. per cent, and the pH 6.89. The administration of insulin and sugar resulted in a rapid recovery, in this instance within 24 hours. In the 19th and 20th weeks insulin was again removed and the animal permitted to eat and again it was noted that only a slow rate of acetone body

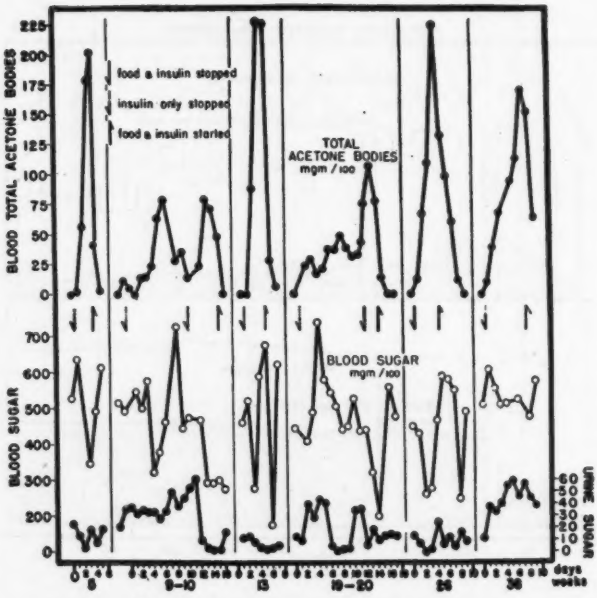

Fig. 4. INFLUENCE OF FOOD AND INSULIN DEPRIVATION of the depancreatized monkey depicted in figure 3.

accumulation occurred in the blood. When food was stopped, the acetonemia rose more rapidly. In the 26th week the response to both insulin and food deprivation was again studied and results essentially similar to those described above were obtained. The D:N of the untreated monkey was 3:1. Of interest is the fact that a study performed in the 38th postpoerative week, in which insulin alone was removed, showed a rising acetonemia which took 6 days to reach a maximum. Restitution of the insulin resulted in a rapid recovery. Thus, so long as food was administered, even in the absence of insulin, the blood sugar was maintained at the high level and the rate of acetone body accumulation in the blood was markedly retarded. Severe acidosis and coma occurred only when both insulin and food were removed. It is noteworthy that the acetonemia consequent to the deprivation of insulin alone is somewhat similar to that observed when food is removed from the normal monkey.

The rapidity with which acetonemia, acidosis, and the associated symptoms occur when the animal is deprived of both insulin and food is depicted in figure 5, constructed from the data of two other animals, one of which had a CO_2 content of 23.1

vol. per cent and a pH of 7.09. One of these monkeys was permitted to remain in coma for 12 hours and then treatment was instituted, but to no avail. In spite of the fact that acetonemia was decreased after glucose and large amounts of insulin were administered, recovery did not ensue.

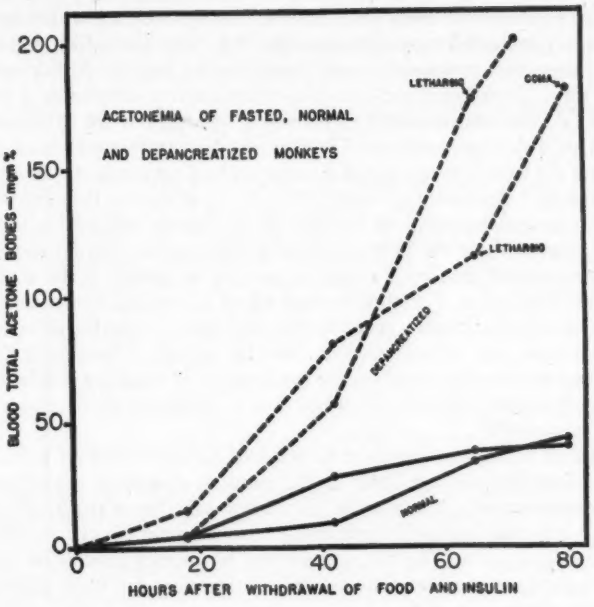

Fig. 5. Influence of food and insulin withdrawal on the development of acetonemia and coma in the depancreatized monkey.

The usual treatment of these episodes of severe acidosis consisted in the administration of insulin at approximately 4-hour intervals together with the intravenous administration of glucose until such a time as when the animal would take food by mouth. This usually occurred in from 6 to 10 hours after the beginning of the treatment. The dosage of insulin employed was from 15 to 25 U for the first 4 hours and 5 U every 4 hours thereafter.

DISCUSSION

It is apparent from these studies that pancreatectomy of the monkey results in the development of a metabolic syndrome which is analogous to that observed in other susceptible species, and which in many respects resembles the clinical syndrome of diabetes mellitus of man. Thus, in contradiction to the descriptions given by previous workers, these animals maintained a high blood sugar, a normal body weight and a glycosuria so long as they are given an adequate diet and adequate amounts of insulin. With an increase in the carbohydrate intake there was also an increase in the glucose excretion. Acetonemia and acetonuria were not apparent during periods in which the animals received adequate therapy, but with the deprivation of insulin, acetonuria did appear. It was also evident that the amount of carbohydrate ingested and consequently utilized was an important factor in the ketogenesis that ensued after

insulin deprivation. When the animals received adequate amounts of carbohydrate, the ketogenesis developed very slowly and usually did not reach very high levels. When the carbohydrate intake was eliminated completely a very severe acetonemia and acidosis ensued. Such observations are in accord with similar findings in man where it has been noted that some patients will develop a very severe acetonemia, acidosis, and subsequently coma when both insulin and food are withdrawn. On the other hand, it has also been noted in man that there are cases which do not develop a rapidly progressive acetonemia even though insulin may be withdrawn for long periods of time. These latter patients do develop a severe acetonemia if food is also withdrawn (4). The same sequence also occurs in the dog, but not to the same degree for in that animal acetonemia never reaches the levels observed either in man or monkey, and the typical symptoms of diabetic acidosis are rarely if ever observed. In the dog it has been demonstrated that, in the absence of insulin, the administration of carbohydrate in amounts adequate for glycogen deposition will inhibit acetone-body formation, presumably in the liver and thereby eliminate an existing acetonemia (5). In the depancreatized monkey the same apparently is possible if the animal is permitted to take food *per os*. The rapidity with which acetonemia ensues consequent to food deprivation in the monkey suggests that this animal's reserves of hepatic glycogen, as in diabetic man (6), are relatively low. By analogy, it is possible that simple pancreatic diabetes in man resembles the syndrome of the monkey, while those cases which develop severe acidosis even when food is administered are complicated by some other abnormality.

It is difficult to account for the data obtained by other workers who could not produce a severe diabetic syndrome in the monkey. Assuming the existence of a complete pancreatectomy, it is possible that if raw pancreas or pancreatin is not administered, a loss of weight and consequently a loss of precursors of carbohydrate and acetone bodies may ensue. The fact remains that when such animals are maintained on adequate diets and receive adequate clinical care, they can be as diabetic as the severest cases observed in man.

SUMMARY

The pancreas was removed from monkeys and the typical syndrome of diabetes mellitus ensued. Such monkeys maintained with insulin remained in perfect health for over one year. When insulin administration was stopped but the animals permitted to eat, only a mild ketosis occurred. However, when both insulin and food were stopped, a severe ketonemia ensued and the associated syndrome of diabetic coma was observed.

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ACTION OF CERTAIN ENZYMES, FISH PANCREATIC ISLETS AND FETAL CALF PANCREAS ON INSULIN

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THERE has not been sufficient evidence reported to disprove the existence in the insulin molecule of a fraction capable, by itself, of lowering blood sugar. Charles and Scott (1), Freudenberg et al. (2), and Fisher and Scott (3) have demonstrated that the destruction of the physiological activity of insulin during enzymatic hydrolysis proceeds at a much greater proportional rate than does the hydrolysis. Although these studies do not offer any evidence for the existence of a physiologically active fraction of insulin, they cannot be interpreted as offering proof that such a fraction does not exist.

Insulin, i.e., the intact protein, has not been demonstrated to be present in any tissue other than pancreatic tissue. The blood-sugar-lowering substance found in other tissues and in the blood may or may not be the same as that found in the pancreas.

We have incubated insulin with enzymes or macerated tissues for periods too short to cause the destruction of all of its physiological activity and then used ultrafiltration to separate any substances with smaller particle size from insulin. Pancreatin was chosen because of the possibility of its containing pancreatic intracellular enzymes, and papain because of its similarity to intracellular enzymes. Fish pancreatic islet nodules and fetal calf pancreas were chosen because of the supposed absence of zymogenous tissue in the former (4) and the indication that tryptic enzymes were absent in the latter until about the fifth month of embryonic life (5). It was thought that these two tissues might contain enzymes produced by the islet tissue capable of releasing a physiologically active fraction from insulin.

EXPERIMENTAL PROCEDURE

The experiment work was done in three parts. In the first part pancreatin and papain were used. Four mg. of crystalline insulin[1] were dissolved in 20 cc. of a McIlvaine sodium phosphate-citric acid buffer at a pH of 7.4. To this were added quantities of enzyme solution varying from 1 to 4 cc. containing from 0.001 to 0.2 mg. of pancreatin or from 2.0 to 6.0 mg. of papain. Difco 1:25 U.S.P. XI Pancreatin and Eimer and Amend 1:200 Mexican Papain were used.

When the activators, cysteine hydrochloride or ferrous sulphate, were used they were added to the enzyme solution and the mixture incubated for one hour previous to its addition to the insulin solution. The quantities of activators used were in all experiments similar to those shown in table 1. Incubation was at 37°C. in the 14 pancreatin experiments and at 55 to 60°C. in the 8 papain experiments. Incubation periods of 4 to 5 hours were used except in one papain experiment in which a 12-hour period was used.

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[1] The crystalline insulin was kindly supplied by Eli Lilly and Company, Indianapolis, Ind.

Ultrafiltration of the solutions after incubation was carried out with acetic acid-collodian membranes prepared by the method of Bechhold and Gutlohn (6), using Bechhold-König crucibles. Membranes were prepared from a solution containing 5 per cent Parlodian (purified Pyroxylin) in glacial acetic acid. Filtration was carried out with a reduced pressure of about 700 mm. of Hg. Preliminary tests had shown that insulin dissolved in the same buffer and in the same concentration as described above would not pass 2, 3, 4, 5, 6 or 7 per cent membranes. When the same concentration of Bacto-peptone dissolved in the same buffer was filtered through these membranes the filtrate gave a positive biuret test in each case. The 5 per cent membranes were chosen for the work because of the fragility and consequent uncertainty of the 2, 3 and 4 per cent membranes which have large pore sizes.

In testing for physiological activity rabbits weighing between 1500 and 2500 gm., previously fasted for 24 hours, were used. Control animals were injected subcutaneously with 0.5 cc. of the unfiltered digest and experimental animals with 6 to 12 cc. of the ultrafiltrate. Blood-sugar determinations were made by the method of Shaffer, Hartman and Somogyi (7, 8). Blood samples were taken from a marginal ear vein before and one and one-half hours after the injection.

Experimental conditions and results of some representative experiments are shown in table 1.

TABLE 1. ACTION OF ENZYMES ON INSULIN

Experiment	Amount of Enzyme, mg.	Cysteine Hydrochloride, Parts per 1 Part of Enzyme	Ferrous Sulphate, ($FeSO_4 \cdot 7H_2O$), Parts per 1 Part of Enzyme	Incubation Period, Hours	Rabbit	Blood Sugar, mg. %	
						Before injection	1.5 hours after injection
Pancreatin							
5	0.025	None	None	4	Control	94	58
					Experimental	98	109
6	0.050	None	None	4	Control	105	120[1]
					Experimental	96	154
9	0.035	1	1.5	4.5	Control	89	51
					Experimental	93	71
11	0.100	1	1.5	5	Control	105	53[2]
					Experimental	103	102
Papain							
17	6	0.1	None	12	Control	94	67
					Experimental	105	118
18	4	0.2	0.3	4	Control	87	49
					Experimental	96	96

[1] The trypsin present destroyed all of the insulin activity.
[2] The cysteine evidently inhibited the trypsin activity.

Experiments with Fish Pancreatic Islet Tissue[2]

In the second part of the work insulin was incubated with pancreatic islet nodules from the Sculpin (Myoxocephalus octodecimspinosis).[3] The islet nodules had

[2] This part of the work was done with the technical assistance of Mr. A. Douglas McLaren.
[3] We are indebted to Dr. A. H. Leim of the St. Andrews, New Brunswick Biological Station for removing the nodules from the Sculpin and shipping them to us.

been frozen with dry ice immediately after killing the fish. They were shipped and kept in dry ice for several days before use.

In the experiments in which cysteine hydrochloride was not used 4 mg. of crystalline insulin was dissolved in 8 cc. of M/15 disodium phosphate and 2 cc. of M/15 monopotassium phosphate added. This buffer mixture has a PH of 7.4. To this solution was added an islet nodule which had been ground with a mortar and pestle while frozen with dry ice. The nodules weighed approximately 100 mg. each. In the experiments in which cysteine hydrochloride was used the 4 mg. of insulin were dissolved in 5 cc. of buffer solution and the cysteine hydrochloride was dissolved in 5 cc. of buffer solution. The nodule was incubated with the cysteine at 38°C. for one hour then the insulin solution added and the whole incubated at 38°C. again. The quantities of cysteine hydrochloride used and the time of incubation of the tissue with insulin are shown in table 2.

TABLE 2. ACTION OF FISH PANCREATIC ISLET TISSUE ON INSULIN

Experiment	Cysteine Hydrochloride, mg.	Incubation Period, hours	Rabbit	Solution Injected cc.	Blood Sugar, mg. %	
					Before injection	1.5 Hours after injection
1		4	Control	0.21	95	33
			Experimental	8.00	87	97
2	0.1	3	Control	0.15	87	16
			Experimental	6.00	71	88
3	2.0	3	Control	0.15	85	37
			Experimental	6.00	65	86
4		1	Control	0.10	102	46
			Experimental	6.00	80	92
5	25.0	3	Control	0.10	92	97[1]
			Experimental	6.00	89	98
6		24	Control	0.10	85	82[2]
			Experimental	6.00	89	103
7	5.0	24	Control	0.10	98	89
			Experimental	6.00	98	96

[1] The cysteine destroyed the insulin activity.
[2] The tissue destroyed the insulin activity on long incubation.

The ultrafiltration of the digests and the tests for blood-sugar-lowering activity were carried out as described above for the pancreatin and papain experiments. The rabbits weighed between 1800 and 2900 gm. The volume of solution injected is shown in table 2, which includes the results of all of our experiments using fish islet tissue.

Experiments with Fetal Calf Pancreatic Tissue

In the third part of the work insulin was incubated with fetal calf pancreatic tissue in 20 experiments. The pancreas was removed from the calf within 2 hours after the death of the cow and kept in a refrigerator between 0 and 5°C. for not more than 2 days. An amount of 8.3 mg. of crystalline insulin were dissolved in 4.0 cc. of M/15 disodium phosphate and 1.0 cc. of M/15 monopotassium phosphate added to give a PH of 7.4. Approximately 200 to 300 mg. of macerated fetal calf pancreas was added and the mixture incubated at 37°C. for periods varying from 0.5 to 10 hours.

When enzyme activators were added they were put in solution in M/15 monopotassium phosphate in concentration such that 1 cc. contained the desired amount of activator. Then 1 cc. of this solution was used instead of 1 cc. of monopotassium phosphate solution.

Ultrafiltration was carried out in this case by the method of McBain and Steuwer (9). A piece of 300 PT cellophane was soaked for 15 minutes in a zinc chloride solution containing 86 gm. of anhydrous zinc chloride per 100 cc. of solution and then washed for 30 minutes in running tap water. The cellophane was finally rinsed with distilled water. The ultrafilters were made of brass and coated inside with paraffin. The membrane was supported by a piece of Whatman #50 filter paper resting on an aluminum plate with fine holes. A pressure of 1500 ± 10 mm. Hg. was used for filtration. Seven hundred and twenty mm. negative pressure was obtained with a suction pump and 780 mm. positive pressure with a column of mercury.

TABLE 3. ACTION OF FETAL CALF PANCREATIC TISSUE ON INSULIN

Experiment	Estimated Age of Fetus, days	Time of Incubation, hr.	Cysteine Hydrochloride, mg.	Ferric sulphate ($FeSO_4 \cdot 7H_2O$) mg.	Rabbit	Blood Sugar, mg. %	
						Before injection	1.5 Hours after injection
4	140	4			Control	102	29
					Experimental	93	92
5	120	9.5			Control	96	45
					Experimental	96	90
9	175	4	0.1		Control	90	38
					Experimental	92	87
11	175	10	0.1	0.1	Control	79	59
					Experimental	105	82
14	200	2.5	0.4		Control	109	43
					Experimental	93	91
15	200	9	0.8		Control	87	30
					Experimental	96	78

As a result of a preliminary series of experiments, using membranes of varying porosity, the 86 per cent membrane was chosen. Under the conditions used it was invariably impermeable to insulin and filtrates from peptone solutions filtered through it always gave a biuret test. A new membrane was prepared for each experiment.

Physiological activity was tested by injecting subcutaneously into the control rabbits 0.04 cc. of the unfiltered solution and into the experimental animals 1.0 cc. of the ultrafiltrate. The animals weighed between 1500 and 2800 gm. Blood samples were taken and sugar determinations made as described above. Some representative experiments are listed in table 3.

DISCUSSION

Since we obtained no blood-sugar-lowering effect with any of the filtrates it is our opinion that these experiments, like those of Charles and Scott (1), Freudenberg et al. (2) and Fisher and Scott (3) cast further doubt on the existence of a fraction of the insulin molecule which is active by itself, but that they cannot be considered as conclusive evidence for the non-existence of such a fraction.

We have made blood-sugar determinations on rabbits at one-half hour intervals

after injecting insulin and as a result of these trials and previous work by others, we chose a 1.5-hour interval. With small doses of insulin, e.g., .5 unit, the blood sugar is at the lowest point at approximately 1.5 hours after injection. With larger doses the lowest level occurs later, but a sample taken at 1.5 hours shows a level below normal. We do not consider a decrease in blood sugar to be significant unless the level drops below 70 mg. per cent. Fasted, untreated rabbits occasionally have blood-sugar levels of 70 mg. per cent, as determined by Shaffer, Hartman and Somogyi method, or even a little below this value. We feel that much work on insulin has been misinterpreted because a drop of 30 or 40 mg. per cent to a level of 70 or 80 mg. per cent was taken as an indication of the presence of a blood-sugar-lowering agent in injected solutions. In our experience with bleeding rabbits from a marginal ear vein, the animals often show a considerably higher blood-sugar level the first time bled than at a bleeding an hour or two later, probably due to greater excitement at the time of the first bleeding.

Seymour (10) showed that ultrafiltration membranes prepared by the method of McBain and Steuwer (9) are isoporous and reproducible and are therefore suitable for ultrafiltration of small colloidal molecules. We have found these membranes much more reliable, i.e., reproducible, than the acetic acid-collodian membranes of Bechhold.

SUMMARY

Insulin was incubated with pancreatin and with activated papain for periods too short to destroy all of its blood-sugar-lowering activity and the digest filtered through an acetic acid-collodian ultrafiltration membrane of definite porosity which was not permeable to insulin. The filtrate was found to have no blood sugar lowering effect.

Insulin was incubated with macerated pancreatic islet tissue from the Sculpin and the mixture ultrafiltered in the same way. The filtrate was without blood-sugar-lowering effect.

Insulin was incubated with macerated fetal calf pancreatic tissue and the mixture filtered through a cellophane membrane of definite porosity, which was impermeable to insulin. The filtrate was found to have no blood-sugar-lowering effect.

These results cast further doubt on the existence of a fraction of the insulin molecule which is capable of lowering blood sugar by itself.

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EFFECTS OF PSEUDOHYPOPHYSECTOMY (UNDERFEEDING), STARVATION, HORMONES AND AGING UPON THE ASCORBIC ACID CONTENT OF THE ADRENAL GLANDS AND LIVER OF THE RAT

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So-called stresses of various kinds produce adrenal enlargement (1). Prolonged complete starvation also results in an increase in the size and weight of the adrenal glands of rats of both sexes. Induced for periods of from 2 (2) to 4–11 (3) days, starvation has been reported to have little or no effect upon the concentration of ascorbic acid in the liver (2–5) or of the adrenals (3, 4).

Dosne and Dalton (6) reiterate Selye's claim (1) that hypertrophy of, and loss of lipoid from, the adrenal glands is the result of requirement of 'excessive amounts of cortical hormones' during 'an alarm reaction' such as severe cold or injections of formaldehyde. Miller and Riddle (7) confirm this in their work on pigeons, but Flexner and Grollman (8) using the reduction of osmic acid as an indicator of adrenal cortical activity in the rat, arrived at opposite conclusions. An adrenal cortical hypertrophy with loss of lipoid similar to that from various 'stress' conditions also occurs during prolonged starvation and may have a similar etiology (9). Oleson and Bloor (10) found that guinea pigs fasted for 7 to 11 days showed a decrease in the cholesterol and cholesterol ester content of the adrenal glands with no fall in the weights of the glands. They believed that this may indicate an increased cortical activity.

Chronic inanition has been found to result in a diminution of the weight of the adrenal glands and of other endocrine organs in a manner qualitatively similar to that following hypophysectomy. The shrinkage in size of the adrenal glands is due to atrophy of the cortex, the medulla remaining virtually unaffected (11). The similarity of the effects of hypophysectomy and chronic underfeeding upon certain of the endocrine organs is due to the fact that inanition in the rat causes depression of the anterior pituitary gland (12). It has also been found that after hypophysectomy there is a marked decrease in the concentration of ascorbic acid in the organs of the rat (13, 14). The smaller size of the adrenal cortex in hypophysectomized animals (15) or during chronic inanition (9, 12) is probably associated with the absent or diminished hormonal function of the hypophysis. Giroud and his coworkers (16) have compared the hormonal potency of the adrenal cortex with its ascorbic acid content and have suggested that the two vary in direct proportion and that, therefore, the ascorbic acid content of the adrenal may be taken as an index of its physiological activity. In a study of the distribution of ascorbic acid in the adrenal glands of horses, pigs, cows and sheep they (17) found that the concentration of ascorbic acid was higher in the cortex than in the medulla of the adrenal gland, a fact which is probably true for the rat as well. In the adrenal gland of the horse the hormonal and ascorbic acid concentrations are lowest in the glomerular zone (18).

The object of the present investigation was to determine the effect of complete

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starvation, of chronic inanition and of old age upon the ascorbic acid content of the adrenal glands and of the liver of the albino rat, and to relate it with the changes of the weight of these organs. The study includes the effects of certain tissues and hormones upon the ascorbic acid content of these two organs. In this way additional evidence was obtained to corroborate the previously observed parallelism between the effects produced by chronic inanition (pseudohypophysectomy) on the one hand and hypophysectomy on the other.

EXPERIMENTAL PROCEDURE

Five sets of experiments were performed. *a*). Rats were subjected to starvation by withholding all food and water for 7 days, when autopsy was performed. *b*). Rats were subjected to chronic inanition for periods of 2 to 7 months by limiting the daily intake of food to one-half of the amount which had been consumed during a preliminary control period. *c*). Normally fed rats receiving food *ad libitum*, were usually run in parallel with the above experimental rats and served as controls. The diet and general procedure in the handling of these animals were similar to that described previously (12). *d*). A number of the above described experimental rats were subjected either to injection of desoxycorticosterone acetate or to implantation of whole endocrine glands. The details of these experiments are given in the text. *e*). To show the influence of age, a group of 42 rats between the ages of 16 and 27 months were autopsied.

At the end of the experimental period the animals were lightly anesthetized with ether and exsanguinated from the heart. The adrenal glands and liver were weighed rapidly and then placed on solid carbon dioxide for a period just long enough to produce complete freezing. The frozen tissue was ground with sand and metaphosphoric acid, centrifuged and the supernatant fluid immediately titrated with a freshly prepared and standardized solution of 2, 6 dichlorophenolindophenol. The total interval from the time of autopsy to the time of titration did not exceed 20 minutes for the adrenals and 45 minutes for the liver. The results obtained by this method were verified in several cases by the method of Mindlin and Butler (19) using a photoelectric colorimeter, and applying a correction for turbidity as suggested by Bessey (20) and for non-ascorbic acid reducing substances by the extrapolation procedure described by Evelyn et al. (21). Only the reduced form of ascorbic acid was determined, usually on the organs of individual rats. For the purpose of comparison, the analyses of the tissues of the experimental rats and of the corresponding controls, were performed on the same day.

RESULTS

Complete starvation. Comparison of the data obtained from female and male rats starved for 7 days, and from groups of normally fed controls indicated that starvation resulted in a reduction of the ascorbic acid content of the liver which was proportional to the fall in liver weight (approximately 40 per cent).

In contrast to the liver, the adrenal glands of the starved rat showed an increase both in size and in ascorbic acid content. In the female rat complete starvation for 7 days resulted in an increase of 38 per cent in the weight of the adrenal glands and an increase of 36 per cent in the content of ascorbic acid. In the male animals a similar period of starvation resulted in an increase in the weight of the adrenal glands of 22 per cent and an increase of 15 per cent in the content of ascorbic acid. Despite a marked difference in the size of the adrenals of normal male and female rats, a fact which is not generally appreciated, the ascorbic acid content of the glands is the same in the two sexes. Statistical examination of the data revealed no significant difference in the concentration of ascorbic acid in the adrenal glands of the completely starved and of the control rats.

We believe that the enlargement of the adrenal glands in complete starvation is not due to hyperemia or edema, because such 'growth' would be accompanied by an evident lowering of the ascorbic acid content rather than by the increase which was observed. This is true because the concentration of ascorbic acid in the blood (approximately 0.01–0.02 mg. per gm.) is much lower than in adrenal tissue (22, 23). An increase in the weight of the adrenal glands, which is accompanied by a proportionate rise in the content of ascorbic acid, would suggest that the enlargement is in great part true physiological growth. This growth may be indicative of an increase in cortical activity (1, 10, 17). The fact that the concentration of ascorbic acid remains unchanged during starvation is interpreted to mean that normally the cells of the adrenal cortex contain a maximum of ascorbic acid. In order to increase the amount, the glands must increase in size.

Chronically underfed rats. Both female and male chronically underfed rats showed a lower content and a lower concentration of ascorbic acid in the analyzed organs than did the normally fed controls (table 1). The lower ascorbic acid content of the adrenals during chronic inanition is probably indicative of decreased cortical activity (13, 14, 17). It has been reported that hypophysectomy resulted in a decrease of the concentration of ascorbic acid in the organs of the rat (13, 14). It was shown previously that during inanition there is hypofunction of the hypophysis, resulting in endocrine effects qualitatively similar to those resulting from hypophysectomy (9). This hypothesis receives further support from the finding described in this report.

TABLE 1. ASCORBIC ACID CONTENT OF ADRENALS AND LIVERS OF CHRONICALLY UNDERFED (C), COMPLETELY STARVED (S) AND NORMALLY FED (N) RATS

Number and Sex	Weights of Rats, gm.		Age, Months at Autopsy	Nutritional Condition	Adrenals			Liver		
	Initial	Final			Wt., mg.	Ascorbic acid concentration		Wt., gm.	Ascorbic acid concentration	
						mg. per gm.	σ		mg. per gm.	σ
Female										
16	199	138	6	C for 69 days	28.5	2.78	0.11	4.15	0.11	0.01
17	199	126	6	S for 7 days	51.3	3.75	0.13	3.37	0.19	0.02
13	184	198	6	N	37.8	3.72	0.16	5.97	0.18	0.01
Male										
8	195	140	4.5	C for 63 days	22.7	3.52	0.14	4.00	0.11	0.01
7	237	183	4.5	S for 7 days	34.1	4.40	0.22	3.86	0.17	0.01
7	196	263	4.5	N	27.9	4.65	0.10	8.07	0.22	0.01

Effect of injections of adrenal gland emulsions. A group of normally fed female rats received subcutaneously 12 daily injections of freshly removed and ground rat adrenal glands from male donors. Two adrenals were used for each injection. A control group was injected with similarly prepared emulsions from the brain tissue of the same donors. Both groups showed severe local reactions to the injections; some became infected and at autopsy showed localized pus. Table 2 shows that in both groups of injected rats the weight of the adrenal glands and their content of ascorbic acid were higher than in non-injected controls as shown in table 1. As for the completely starved rats, the concentration of ascorbic acid did not change. It had been noted previously that when normally fed or completely starved rats were injected with tissue emulsions which caused a local or generalized reaction, but which were otherwise hormonally inactive, there resulted an enlargement of the adrenal glands. The enlargement was similar to that resulting from complete starvation. In the pres-

ent experiments there was found an increase both in the weight and in the ascorbic acid content of the adrenal glands of the rats which had received either adrenal gland injections or brain injections, and it was concluded that the reaction could not be attributed to some specific hormonally active substance present in the tissue suspensions. Following these injections, the ascorbic acid content of the liver decreased.

Effect of desoxycorticosterone acetate.[1] Two groups of normally fed female rats, one group of normally fed male rats and one group of chronically underfed female rats received daily injections of 0.25 to 1.0 mg. of desoxycorticosterone acetate in sesame oil (10 mg. per cc.) for periods of 1 to 2 months. Corresponding control groups received injections of plain sesame oil for similar periods of time. The data obtained for the controls and for the groups treated with desoxycorticosterone acetate indicate that the dosages of desoxycorticosterone acetate used in these experiments (which would be sufficient to maintain life in adrenalectomized rats) influenced neither the ascorbic acid content nor the weight of the adrenal glands or the liver of the rat (table 2).

TABLE 2. ASCORBIC ACID CONTENT OF ADRENAL GLANDS AND LIVERS OF TREATED RATS

Number and Sex	Weight, gm.		Age, Months at Autopsy	Nutritional Condition[1]	Treatment	Adrenals			Livers		
	Initial	Final				Wt., mg.	Ascorbic acid concentration mg. per gm.	σ	Wt., gm.	Ascorbic acid concentration mg. per gm.	σ
Female					Injections of:						
4	211	206	6	N	Adrenal gland emulsions	52.5	3.71	0.12	8.38	0.11	0.01
4	207	205	6	N	Brain tissue emulsions	51.0	3.57	0.09	6.52	0.12	0.01
3	5	117	2	N	Injections of DCA[2a]	18.4	4.98	0.08	4.82	0.19	0.01
3	5	115	2	N	Sesame Oil Control	18.4	4.73	0.18	4.76	0.17	0.01
4	43	161	3	N	Injections of DCA[2b]	22.7	3.47	0.27	6.20	0.15	0.01
4	44	153	3	N	Sesame Oil Control	26.8	3.25	0.08	5.78	0.16	0.01
Male											
4	48	239	3	N	Injections of DCA[2b]	27.3	3.12	0.02	9.62	0.20	0.00
4	48	229	3	N	Sesame Oil Control	25.4	3.08	0.04	8.54	0.22	0.01
Female											
5	196	141	11	C, 185 days	Injections of DCA[2c]	25.3	2.96	0.07	4.24	0.09	0.00
5	198	138	11	C, 185 days	Sesame Oil Control	25.4	2.88	.05	4.40	0.09	0.00
Male											
5	203	139	7	C, 149 days	13 pituitary implants	29.7	2.70	0.07	4.51	0.08	0.01
4	199	143	7	C, 149 days	13 brain tissue implants	21.6	2.92	0.01	4.38	0.09	0.01
Female					*Ovariectomized*						
4	199	136	12	C, 227 days	22 pituitary implants	37.4	3.76	0.00	4.32	0.11	0.00
4	197	139	12	C, 227 days	22 brain tissue implants	26.8	2.62	0.01	4.00	0.09	0.00

[1] Normally fed (N), Chronically Underfed (C)
[2] Desoxycorticosterone Acetate: (a) 0.25 mg. per day for 2 months
(b) 0.30 mg. per day for 2 months
(c) 1.00 mg. per day for 1 month

Effect of implants of pituitary glands. Each of 4 female (ovariectomized) and 5 male chronically underfed rats received intramuscular implants of whole pituitary glands from normally fed donors. Concomitantly, 4 female castrates and 4 male chronically underfed control animals received implants of brain tissue from the same donors. There was no evidence of infection or of inflammation at the site of injection in this group of rats. Despite the continued underfeeding, the male rats which received pituitary implants had adrenal glands which were larger than those of the brain-injected controls (table 2) and equal in size to those of normally fed male rats (table 1). The ascorbic acid content of the adrenal glands of the underfed rats which

[1] The desoxycorticosterone acetate used in these experiments was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

received pituitary implants increased proportionately to the rise in the weight of these organs.

Ovariectomized, chronically underfed rats implanted with pituitary glands had adrenals which in size and in ascorbic acid content were equal to those of normally fed unoperated female rats. The content of ascorbic acid of the adrenal glands of the rats which had received pituitary implants was double that of the control group receiving brain implants, while the concentration was but 44 per cent higher. The increase in the size of the adrenal glands of the female rats which received pituitary implants could not have been due to the presence of the sex hormones incident to the pituitary implants, but to a direct effect of the implants upon the adrenal cortex.

Chronic inanition, as well as hypophysectomy, results in a lowering of the weight and of the ascorbic acid content of the adrenal glands of the rat. Implantation of pituitary glands into chronically underfed rats increases the weight and the ascorbic acid content of the adrenal glands. It has been shown that the fall in the weight of the adrenal glands during chronic inanition as well as after hypophysectomy is due to a decrease or a loss of pituitary function since in both cases the adrenal glands may be stimulated by pituitary implants. One can conclude, therefore, that the fall in the concentration of ascorbic acid of the adrenal glands of the chronically underfed rat is due in part to malnutrition itself, but in greater part to a fall in adrenotropic hormone concentration. This is secondary to a depression in the function of the pituitary gland resulting from the inanition (pseudohypophysectomy).

Implantation of pituitary glands into chronically underfed rats had little effect on the size or ascorbic acid content of the liver. It appears, therefore, that the fall in the concentration of ascorbic acid of the liver of the chronically underfed rat is not a reflection of the depressed hypophysis but is due either to the malnutrition, or to a depression of some other secretion. The excessive fall in ascorbic acid in the liver of hypophysectomized rats reported by Leblond (14) and Bowman (13) would, however, suggest a hypophyseal relationship similar to that for the adrenal glands.

Effect of age. Normally fed rats of 16 to 27 months of age showed a decrease in the concentration of the ascorbic acid of the adrenal glands and of the liver (table 3). In the adrenal glands of these animals the concentration of ascorbic acid reached levels as low as and sometimes lower than those of the younger (about 5 months) but chronically underfed rats (compare table 3 with tables 1 and 2). In the liver the concentration of ascorbic acid was lowered by aging, but was considerably less than that by chronic underfeeding, while there was a marked increase in the weight of the liver and in body weight.

These results bring additional evidence to the previously made observation that

TABLE 3. INFLUENCE OF AGE ON WEIGHT AND ASCORBIC ACID CONTENT OF ADRENALS AND LIVER OF THE NORMALLY FED RAT

Number and Sex	Weight, gm.	Age, Months at Autopsy	Adrenals			Liver		
			Weight, mg.	Ascorbic acid concentration		Weight, gm.	Ascorbic acid concentration	
				mg. per gm.	σ		mg. per gm.	σ
Female								
13	198	5	37.8	3.72	0.22	5.96	0.18	0.01
2	261	27	46.0	2.67	0.10	8.25	0.14	0.00
Male								
7	263	5	27.9	4.59	0.10	8.07	0.22	0.01
30	338	16	34.0	3.67	0.14			
4	418	21	35.8	3.04	0.08	12.79	0.17	0.01
6	373	27	34.8	2.80	0.07	11.00	0.17	0.01

the direct effect of underfeeding is only a minor factor in influencing the concentration of ascorbic acid in the adrenal glands of the rat, while it is evidently the main factor affecting the concentration of the vitamin in the liver. It is suggested that in old rats there is a diminished function of the pituitary gland similar to that found from under-nutrition in younger animals. The effects from under-nutrition or old age are similar and only quantitatively lower than those which obtain after complete ablation of the hypophysis (13, 14).

SUMMARY

1. The ascorbic acid content of the adrenal glands and of the liver of rats was determined upon chronically underfed (pseudohypophysectomized), completely starved, normal control rats, and upon a group of aged rats.

2. *a*). The adrenal cortical atrophy which was present in chronically underfed rats was accompanied by a fall in the ascorbic acid content and concentration.

b). The loss in weight of the liver was proportional to the loss in body weight so that its ascorbic acid concentration did not change.

3. Pituitary implants injected into chronically underfed rats resulted in a proportionate increase in the weight and the ascorbic acid content of the adrenal glands.

4. *a*). During complete starvation there was an increase in the weight and in the ascorbic acid content of the adrenal glands, which was greater in the female than in the male rats.

b). There was a fall in the weight of the liver and a decrease in the ascorbic acid content proportional to the fall in body weight.

5. The increase in weight of the adrenal glands which accompanies the administration of non-specific toxic substances (tissue emulsions) is accompanied by a rise in the weight and in the ascorbic acid content of the adrenal glands.

6. Desoxycorticosterone acetate in oil injected in daily doses of 0.25 to 1.0 mg. for periods of 1 to 2 months altered neither the weight nor the ascorbic acid content of the adrenal glands of young normal rats.

7. Normally fed rats of 16 to 27 months of age, when compared with younger rats, showed a decrease in the concentration of the ascorbic acid of the adrenal glands and of the liver.

8. The data obtained for chronically underfed rats (pseudohypophysectomized) and for those reported to follow hypophysectomy is further evidence for the qualitative similarity between these two conditions.

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NOTES AND COMMENTS

RUSTING IN DESALIVATED ALBINO RATS*

THE SUSCEPTIBILITY of the albino rat to experimental dental caries has encouraged the use of this animal in laboratory investigations of the causes of tooth decay. Recently it has been shown that the extirpation of the salivary glands hastens the onset of the carious process and increases its severity. If desalivated rats are to be used in the study of the factors concerned with the etiology of dental caries, it would seem pertinent to investigate the effect of salivary gland extirpation on the general development of the rat. Preliminary studies in this direction have indicated that there is a possible relationship between the physiology of the salivary glands, the vitamin B complex and the sex hormones.

Experimental procedure. Thirty-six albino rats from litters of inbred stock were divided into 3 comparable groups, each containing 6 males and 6 females. At weaning all of the animals were placed on a liquid diet containing equal parts of distilled water and irradiated evaporated milk, adequately fortified with copper, iron and manganese.[1] *Group I* animals served as controls. *Group II* animals had a sham operation in which the skin above the salivary glands was incised, the parotid, submaxillary and sublingual glands were exposed and handled but left intact, after which the opening was sutured. *Group III* animals had the above mentioned salivary glands removed. Detailed information concerning food consumption, growth, weight, maintenance, basal metabolic rate and other factors will be reported elsewhere.

It will suffice to say that the data reported for *Groups I* and *II* are quite comparable and that the male rats in *Group III* showed a slight decrease in growth, and a moderate rusting of the fur. The changes in the female desalivated rat were much more striking. There was a significant reduction in the rate of growth, that is apparently not related to the food consumption, and a pronounced rusting of the fur after 3 to 6 weeks on the diet. Although the rusting showed a progressive increase in intensity with age, the general health and activity of the animals was not markedly impaired. Daily injections of 40 micrograms of pantothenic acid and 20 mg. of choline[2] resulted in a considerable improvement of the condition but did not affect a complete cure.

Discussion. Rusting in albino rats has been related to *a*) deficiency in the vitamin B complex†,[3] *b*), deficiency in dietary copper[4] and *c*), disturbances in the metabolism of sex hormones‡.[5] Since the milk diet was fortified with copper, it is probable that we are not dealing with a condition resulting from a deficiency of this element. In addition, the deficiency did not manifest itself in the animals of *Group I* and *II* which were maintained on the same diet. The experimental evidence is much more suggestive of *a*), a vitamin B-complex deficiency, since injections of pantothenic acid and choline alleviated the condition, or *b*), a disturbance in the metabolism of sex hormones, since the condition was by far the more striking in the female rats. There is a strong possibility that both factors are involved since the control

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† The available literature indicates the probability that the greying of piebald rats and the rusting of albino rats observed on vitamin B-complex deficient diets, are comparable.

‡ In these laboratories, W. F. Koss observed rusting of the fur in young male rats receiving 400 R. U. of estradiol benzoate twice a week.

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animals (*Groups I* and *II*), which were raised on the same diet, gave no evidence of the rusting of vitamin B-complex deficiency and since desalivated female rats maintained on other diets, do not show a reddening of the fur. Reports in the literature indicate the inability of animals deficient in vitamin B-complex to mate[6] and a failure in lactation.[7] It has also been shown that there is a decreased fertility of eggs from hens maintained in vitamin B-complex deficiency[8] and that the liver from rats deficient in vitamin B complex is unable to inactivate estrogens.[9] All of these data are evidences of a possible relationship between the vitamin B complex and the sex hormones.

The findings of Higashijo,[10] that removal of the submaxillary glands and ligation of the parotid gland in young rats results in hypertrophy of the uterus and atrophy of the testes point to an endocrine-like behavior of the salivary glands toward the sex organs. The same worker has reported atrophy of the corpus luteum of young rats from which the submaxillary glands have been removed and which are receiving injections of estrone; there is a failure of estrone to produce a similar condition in animals with intact submaxillary glands. Both of these observations suggest the ability of the salivary glands to modify the effect of estrogens. That the reverse is also true is apparent from the work of Lacassagne[11] who has reported that submaxillary glands in male and female mice show a sex-linked histological difference. Upon the administration of testosterone the submaxillary gland of the female mouse changes to the male type and the reverse is produced when the male mouse is given folliculin.

Summary. Rusting of the fur has been observed in desalivated rats maintained on a milk diet. The condition was absent in control, unoperated rats receiving the same diet. The effect was much more pronounced in female than in male rats. It is believed that the condition results from a disturbance of the normal relationship between the vitamin B complex and the sex hormones by the removal of the salivary glands.

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RECONSIDERATION OF THE SIGNIFICANCE OF SOME PLANT CHARACTERISTICS IN RELATION TO YIELD OF GONADOTROPIC MATERIAL*

Extracts have been obtained from dried plant tissue, and from the fresh juice of several kinds of plants, which induce the formation of corpora lutea when injected into estrous rabbits.[1–5] Juices pressed from plants grown under different conditions, and assayed during different times of the year, varied with respect to 'activity' content. Friedman and Mitchell[5] attempted to determine what factors could be correlated with these variations in response, and directed their attention mainly toward the plant, since it was thought that the gonadotropic activity of juice might well vary with the age, stage of de-

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TABLE 1. GONADOTROPIC ACTIVITY OF EXTRACTS FROM JUICE OF CORN AND OAT PLANTS GROWN DURING 1941

Sample Number	Harvest Date	Growing Conditions	pH Fresh Juice	Activity: Ovulating Doses per Liter
		Corn		
141	6/30/41	field	5.5	0[1]
53	7/ 1/41	field	5.4	0
54	7/ 2/41	field	5.4	14
55	7/ 7/41	field	5.3	0
56	7/14/41	field	5.6	0
57	7/30/41	field	5.1	12
241	8/ 5/41	field	5.2	12
341	8/14/41	field	5.1	0
441	9/ 3/41	field	5.4	0
541	9/ 3/41	field	5.4	0
641	9/ 9/41	field	5.3	0
58	9/30/41	field	5.3	0
59	9/30/41	greenhouse	5.3	0
61	12/ 1/41	greenhouse	5.3	0
		Oats		
141	4/23/41	field	6.1	0
241	6/ 9/41	field	6.1	12
341	6/17/41	field	6.0	20
441	6/24/41	field	6.1	25–30
541	8/26/41	field	6.0	0
641	9/ 3/41	field	5.9	0[2]
741	9/ 9/41	field	6.2	0[2]
60	11/12/41	greenhouse	6.0	0
62	12/ 9/41	greenhouse	5.9	0
63	1/ 6/42	greenhouse	6.1	0
64	1/30/42	greenhouse	6.1	0
65	3/10/42	greenhouse	6.1	0

[1] All zero readings indicate less than 10 ovulating doses per liter.
[2] Frozen juice: assayed 100–125 ovulating doses per liter when tested in February and March, 1942.[1]

velopment, and physiological condition of the plant. They reported that the animal responses observed varied with respect to the initial pH of the expressed juice and to the season in which the plants were grown and assayed.

Experiments have subsequently been carried out for the purpose of gathering additional information concerning these variations. The results show more clearly the significance of the data previously obtained by Friedman and Mitchell, and suggest that a re-interpretation of these data would facilitate further work in this field. Fresh juice expressed from the above-ground portions of oat and corn plants was used in these later investigations. The plants were grown under field and under greenhouse conditions. In some few instances slight modifications of the methods of extraction previously described[4,5] were introduced, such as allowing the juice to chill overnight in the cold room as an aid in clarification, and storing portions of the fresh juice in tinned cans in a freezing room for future study.

In the present experiments using fresh juice, positive responses were obtained only during June, July, and August (table 1); and even during these months some samples gave negative results, although the plants were reasonably alike with respect to size, stage of development, and succulency.

The pH of various samples of fresh juice from corn plants varied between 5.1 and 5.6, while that from oats ranged between 5.9 and 6.2 (table 1). These values represent the hydrogen-ion concentration of juice from all the various lots of plants grown during different seasons under widely different environmental conditions, including lots which varied with respect

TABLE 2. GONADOTROPIC ACTIVITY OF EXTRACTS PREPARED FROM JUICE OF CORN AND OAT PLANTS GROWN DURING 1939–1940[1]

Sample Number	Age, Days	Harvest Date	Growing Conditions	pH Fresh Juice	Activity: Ovulating Doses per Liter
			Corn		
2	33	6/21/39	field	5.4	20
11	24	7/ 6/39	field	5.5	0[2]
13	53	7/10/39	field	5.3	0
17		9/18/39	greenhouse		0
18	26	10/30/39	field	5.7	0
40	28	6/24/40	field	5.3	290
41	26	7/ 1/40	field	5.4	15
42	42	7/ 8/40	field	5.3	13
43	42	7/ 8/40	field	5.3	12
44	29	7/10/40	field	5.3	15
46	35	7/16/40	field	5.5	0
49	13	7/23/40	field	5.5	12
50	21	7/23/40	field	5.4	12
51	40	7/23/40	field	5.3	35
52	61	7/23/40	field	5.2	25
			Oats		
15	30	9/ 1/39	greenhouse		0
16		9/18/39	greenhouse		0
19	31	10/30/39	greenhouse	6.5	0
20	59	11/ 6/39	greenhouse	6.3	0
21	59	11/10/39	greenhouse	6.3	0
22	40	11/16/39	greenhouse		0
23	60	11/21/39	field		0
24	75	12/ 5/39	field	6.2	0
27	35	12/13/39	greenhouse	5.9	0
29	47	1/31/40	greenhouse	6.1	0
30		2/20/40	field (Arizona)		0
31	31	4/11/40	greenhouse	6.0	0
32	31	4/11/40	greenhouse	5.9	0
34		6/ 3/40	greenhouse	6.0	0
35	41	6/ 3/40	field	6.0	0
36	33	6/12/40	field	5.9	0
45	36	7/16/40	field	6.0	14
47		7/19/40	field	5.8	0

[1] Previously reported in part 4.
[2] All zero readings indicate less than 10 ovulating doses per liter.

to age, stage of development and physiological condition. It is apparent that the hydrogen-ion concentration of juice from corn was characteristically different from that of juice from oat plants, irrespective of the environmental conditions under which the plants were grown.[6] With respect to gonadotropic activity, there was no apparent correlation between the hydrogen-ion concentration of the fresh juice and the animal response observed.

Friedman and Mitchell[5] found that the PH of corn juice used in their experiments varied between 5.2 and 5.7, while that of juice from oat plants usually ranged between 5.8 and 6.3 but in one instance tested 6.5 (table 2), and this is in agreement with results of the present study. Their data also fail to show that there was any apparent correlation between the hydrogen-ion concentration of the juice and the amount of gonadotropic activity factor obtained.

In summary, it is evident from the data collected during the last 3 years, that ovulation has been induced in rabbits as the result of the administration of some plant juice extracts

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during the spring and summer months as measured by arbitrary standards previously described,[4,5] while many negative results have been observed during all seasons of the year. Positive assays have not been found to be correlated with any of the plant characteristics noted such as age, succulency, hydrogen-ion concentration of the juice, or with any factor of the environments under which the plants have been grown.

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